Enzyme supplementation in poultry feed: *In vitro* and *in vivo* effects on microbial growth

BY

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A Thesis
Submitted to the Faculty of Graduate Studies
In Partial Fulfillment of the Requirements
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Of

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ABSTRACT

The partial ban of antibiotics in animal feed has increased the need for alternative methods to improve and maintain animal health. Although exogenous enzymes have been added to poultry diets for years in order to reduce the viscosity of feed caused by non-starch polysaccharides (NSP), it is now believed that they may also result in health benefits to the animal. In this respect, addition of enzymes may alter the composition or quantity of both natural microflora and pathogenic microorganisms in the poultry gut. Further, it is possible that an alteration in gut bacteria can, in part, contribute to changes in digestibility of feed.

In order to determine the effect of enzyme supplementation on gut bacteria, microorganisms commonly found in the poultry gut (*Enterococcus faecalis, Enterococcus gallinarum, Lactobacillus brevis, Bifidobacterium pullorum, Bifidobacterium gallinarum, Bifidobacterium animalis, Lactobacillus rhamnosis and Lactobacillus acidophilus*) and important pathogenic bacteria (*Listeria monocytogenes, Escherichia coli, Salmonella* Typhimurium, *Salmonella* Pullorum, *Clostridium perfringens* and *Campylobacter jejuni*) were tested for growth on common feed components (β-glucan, xylan, galactomannan and raffinose) with or without addition of the corresponding enzymes (glucanase, xylanase, galactanase and galactosidase). Results of this study showed that those enzymes that released large amounts of free sugars yielded the highest increase of bacterial growth over non-enzyme treated substrates. Selected microorganisms were also tested for growth on soybean and canola meals previously subjected to ethanol-extraction in order to remove simple sugars. *Campylobacter jejuni* was the only microorganism to show increased growth with

inclusion of enzyme for both meals; while only *Escherichia coli*, coliforms, *Lactobacillus brevis* and *Bifidobacterium pullorum* showed an increase in growth with enzyme addition to canola meal. Overall the results indicate that the meals were not being enzymatically degraded to simple sugars where they would be readily used by the majority of microorganisms.

A chicken growth trial was performed using the same enzymes in various cereal based diets (wheat, barley and corn). During the trial, *Escherichia coli*, coliforms, enterococci, aerobic and anaerobic spore-formers were monitored for growth changes in the animal caeca and ileum. *E. coli* numbers in the caeca decreased in enzyme supplemented wheat and corn-based diets, while numbers increased slightly in the barley-based diet. Growth of enterococci increased in the caeca with enzyme addition only for the barley based diet. Spore-formers increased in the caeca with the enzyme for all three diets. Growth changes in the ileum were minimal while bacterial growth occurred in the caeca upon addition of the exogenous enzyme. All coliforms exhibited characteristics of *E. coli* and were as such not enumerated. Treatments involving addition of a two species *Bacillus* probiotic were also included but showed minimal difference in growth, except for a slight increase in numbers of spore-forming bacteria in the corn-based diet. All treatments showed an increase in growth performance with enzyme addition and the majority showed no additional effect with the probiotic.

1.0 INTRODUCTION

The feed industry strives continuously to improve the quality of their products in order to maximize animal performance and overall health. The goal is to create a diet that provides high nutritive value with low waste, yet remain cost effective. The digestive process of animals is far from being fully efficient and therefore waste occurs, decreasing profitability. Exogenous enzymes are used as an extension of the digestive process to increase availability of nutrients and to decrease nutrient excretion into the environment (Sheppy, 2001). Feed components such as wheat, barley or rye contain high levels of non-starch polysaccharides (NSPs) which increase digesta viscosity and reduce the quantity of nutrients absorbed by animals (Sheppy, 2001). For example, studies by Annison (1991) have shown that reductions in the energy levels of various diets fed to poultry are directly related to the levels of water-soluble NSPs present in their diets. This increased viscosity also results in a low rate of feed passage through the intestine leading to an increase in microbial growth (Sheppy, 2001). Therefore, it is likely that the natural microflora of the poultry gastrointestinal tract (GIT) could also be affected by enzyme addition and may be a key factor in improving nutrient utilization (Choct and Annison, 1992). Although the primary purpose of adding exogenous enzymes to poultry diets is to break down NSPs and reduce viscosity to improve digestibility, the relationship between exogenous enzyme supplementation and bacterial growth in the GIT is not clear. Further, altering enteric bacterial populations in the GIT may allow pathogens greater access to nutrients and lead to an increase in their growth.

The basis for exogenous enzyme supplementation is to improve the nutritive value of feed. Enzymes used for this purpose have been demonstrated to break down anti-nutritive factors in feed that cause digestive problems and poor growth. Enzymes can also increase the availability of starches and proteins in feeds that are encapsulated by the plant cell wall structure and allow access by the animal's own enzymes. Furthermore, enzyme supplements can provide young animals with activities complimenting their own immature digestive system (Sheppy, 2001). The more efficiently an animal utilizes feed, the less excreta is produced and this is beneficial to the environment (Sheppy, 2001).

Research suggests that the scale of response to exogenous enzymes seems to depend not only on the initial quality of the feed (types and quantities of NSPs) but also on the microbial status of the bird (Sheppy, 2001). In studies using both enzymes and antibiotics, there was generally a reduced response to enzymes in the presence of an antibiotic. This suggested that the response to enzyme supplementation could be reduced due to impaired growth of gut microflora (Bedford and Apajalahti, 2001). By reducing intestinal microflora proliferation, the beneficial effects of enzyme addition were limited, indicating that gut microflora may have a significant impact on nutrient utilization, probably by increasing the energy extraction from the diet.

A problem in the feed industry is the variability in digestive systems among animal species making it difficult to determine the most efficient diet. Exogenous enzymes can reduce the variability in nutritive value of animal feeds and improve the overall value of feed formulations (Sheppy, 2001). Therefore, inclusion of enzymes in diets could yield better uniformity in growth performance, could support good animal management practices and increase profitability. The use of exogenous enzymes in

animal feeds is increasing due to research data which has given a better understanding of their mode of action and the beneficial effects they may have in the animal gut (Sheppy, 2001). The global enzyme market in 1995 was approximately 1 billon dollars U.S. and it is estimated that it will rise to between 1.7 and 2 billion dollars by 2005 (Sheppy, 2001). This has prompted an increase in research to develop better, more functionally suited enzymes targeted for the hydrolysis of complex and otherwise indigestible feed components.

This study was conducted to determine the effect of enzyme hydrolysis products on the growth of chicken gut microflora. The *in vitro* portion of research involved an examination of the growth of individual bacteria commonly associated with the GIT. Feed substrates consisting of sugar oligomers β-glucan, xylan, guar gum, and raffinose, with or without specific enzyme supplementation, were used to assess bacterial growth. Although the commercial enzymes used in this investigation (glucanase, xylanase, galactanase and galactosidase) have been used to reduce substrate viscosity and increase nutrient absorption, it was of interest to determine if bacterial growth would be affected by the availability of low molecular weight enzyme hydrolysis products. Although valuable information can be obtained from studies of the growth of microorganisms on selected substrates, these do not accurately simulate real-life conditions. In addition, although the substrates used in the *in vitro* study represented some important feed components, the ability of microorganisms to utilize whole feed (soybean, corn and canola based) in the presence of enzyme supplements must also be evaluated. In order to ensure that microbial growth did not result from the metabolism of simple sugars naturally present in feed, ethanol-extraction was performed before enzyme addition. For

these tests a commercial preparation of multi-carbohydrase enzymes was added to the ethanol-extracted meals and growth of individual bacteria was monitored.

The *in vivo* portion of this study involved a poultry growth trial using wheat, barley and corn-based diets with or without exogenous enzymes. Enzymes similar to those used in the *in vitro* study were evaluated. Further, the inclusion of a probiotic cocktail was also assessed with respect to changes in the microflora of the GIT. Alterations in bacterial populations with respect to enterococci, *Escherichia coli*, coliforms, aerobic spore formers and anaerobic spore formers in the caeca and ileum were determined. Body weight gain and efficiency of feed utilization were also monitored. The purpose of this portion of the study was to better understand the effects of exogenous enzyme addition to diets with respect to bacterial changes in the GIT of poultry.

Detailed objectives of this investigation were:

- To assess and compare the growth of various enteric bacteria, including pathogens, using NSP substrates with or without addition of exogenous enzyme.
- To monitor the growth of various enteric bacteria using ethanol-extracted feed ingredients from which simple sugars were removed; with or without exogenous enzymes.
- To determine the effect of exogenous enzymes and probiotic addition, separately and in combination, on growth of selected microbial populations in the caeca and ileum of broiler chickens fed wheat, barley and corn-based diets.
- To determine the effect of exogenous enzymes and probiotic addition, separately and in combination, on the growth performance of broilers.

2.0 LITERATURE REVIEW

2.1 Introduction

Research regarding enzyme supplementation in animal feed has generated mixed and variable results which have led to skepticism and disagreement. Overall, the goal of the poultry industry is to grow a consistent, high quality product in the least amount of time at minimal cost (Acamovic, 2001). Further, producers would like to decrease the amount of waste material for both economic and environmental reasons. One solution is to increase digestibility by using high quality diets with an adequate supply of available nutrients. Feed is estimated at 70-80% of the total cost of rearing poultry and therefore, any method of decreasing cost will have a profound effect for the producer (Acamovic, 2001). Also, for economic reasons it would be beneficial to improve the digestibility of common feedstuffs including cereals such as wheat, rye, and barley. Unfortunately, these cereals contain high amounts of non-starch polysaccharide (NSP) which result in viscous intestinal material contributing to impaired digestion and nutrient absorption. Today exogenous enzyme addition to feed is mainly used to reduce viscosity of digesta, but other benefits have been explored, as indicated by Marquardt (1997) in Table 1. Recently, one of the benefits cited by this author (related to altered populations of microorganisms in the GIT) has received increased attention from the feed industry.

A more direct method to alter the natural microflora in animals involves the inclusion of probiotics. The concept of probiotic use involves addition of live microbial supplements primarily to feed or water in order to improve the overall health of farmed

animals and perhaps replace the need for growth-promoting antibiotics. This review examines the use of both exogenous enzymes and probiotics in poultry feed, what is known and what remains inconclusive.

Table 1. Benefits obtained from the addition of enzymes to poultry feeds.

Reduced viscosity in the diet and digesta
Enhanced digestion and absorption of nutrients especially fat and protein
Improved apparent metabolisable energy value of the diet
Increased feed intake, weight gain, and feed to gain ratio
Reduced beak impaction and vent plugging
Decreased size of gastrointestinal tract
Altered population of microorganisms in gastrointestinal tract
Reduced water intake
Reduced water content of excreta
Reduced production of ammonia from excreta
Reduced output of excreta, including reduced N and P
Reduced output of bile salts in digesta

Marquardt (1997).

2.2 Galactooligosaccharides

Galactooligosaccharides, such as raffinose and stachyose, are commonly found in feed. Raffinose, which is considered in this study, is a tri-saccharide found in whole grains and meals (soybean) as well as many vegetables such as brussel sprouts and beans. It is composed of D-galactose and sucrose which are joined by α (1-6) glycosidic linkages

which can be hydrolyzed by α-galactosidases. Most monogastrics (including poultry) do not possess the α-galactosidase enzyme and therefore these oligosaccharides pass through the stomach and small intestine intact (Merck Index, 2004). Raffinose can be broken down in the large intestine by anaerobic bacteria which produce α-galactosidase, however, raffinose does not increase the viscosity of digesta and therefore when added α-galactosidase would function to produce simple sugars that could be absorbed by the animal.

2.3 Non-Starch Polysaccharides (NSP)

Cereals such as wheat, barley, and rye are commonly used in the feed industry. Unfortunately, they contain high levels of NSP which may exhibit anti-nutritive effects such as poor growth and low nutrient utilization; wheat for example has been shown to contain up to 50-80 g NSP/kg dry matter (Choct and Annison, 1992) or from 8.3 to 9.8% total NSP (Slominski *et al.*, 2000). The major cause of these anti-nutritive effects is believed to be the increased viscosity of the digesta due to compounds including β–glucans and arabinoxylans (Choct, 2001; Annison, 1991). High viscosity digesta results in detrimental physiological and morphological effects on the digestive tract of the animal such as sticky litter or hock burns (Sheppy, 2001). Further, the composition and quantity of bacteria in the GIT of poultry may be affected due to the slowed rate of passage of intestinal contents and physical changes to the intestine itself such as widening (Choct, 2001). A slower rate of passage allows bacteria more time to access nutrients resulting in higher growth levels. The ability of the digestive system to absorb nutrients

is partly dependent upon the microbial species present in the gut as well as their total population. The composition of the microflora is dependent on the diet for its sources of metabolism (Bedford and Apajalahti, 2001) and as such changes in diet will affect the microbial composition of the GIT. Indeed, exogenous enzymes, particularly NSP degrading carbohydrases have not only been shown to improve digestion in poultry but also have been shown to induce changes in microflora populations. Whether this phenomenon is simply an adaptation to a new environment or a physiological response in the host remains unclear (Bedford and Apajalahti, 2001).

2.3.1 β-glucan

Unlike cellulose which consists of straight chain glucose polymers with only β1,4 linkages, barley and oats contain β-glucans (mainly located in the endosperm, aleurone layer and bran) which consist of glucose polymers having a specific mixture of β1,3 and β1,4 linkages (Choct, 2001). Barley contains sufficient amounts of these mixed linkages (3-4%) which results in digesta with higher viscosity, particularly in broiler chicks. This phenomenon contributes to poor or impaired nutrition in poultry since interaction between the feed and digestive enzymes is slowed, resulting in delayed uptake of carbohydrates, amino acids and lipids (Choct, 2001). Hesselman and Aman (1986) and Campbell et al. (1989) reported success using β-glucanase in order to increase growth and digestibility in poultry. However, exogenous enzymes are usually only able to partially digest β-glucans and therefore do not reduce them completely to glucose

(Sheppy, 2001). Further, barley contains other soluble NSPs, and therefore β-glucanases are often used in combination with other carbohydrases such as xylanase (Choct, 2001). Other possible explanations for the positive effects of β-glucanase addition may be related to the increased availability of starch to hydrolyzing enzymes in the intestinal tract due to the breakdown of the endosperm which is known to contain ample amounts of soluble NSPs (Hesselman and Aman, 1986). Enzymatic hydrolysis is especially important in regards to increasing metabolisable energy values since starch is considered to be highly digestible in most birds although the exact effect on adult broilers is not fully known.

Glucanase enzymes are usually added at levels between 0.1 and 0.3% (Malathi and Devegowda, 2001). Although specific activities of the enzymes used are considered more important. A study by Hesselman and Oman (1986) showed increased feed consumption, live weight and improved feed conversion as a result of β-glucanase supplementation. The apparent ability of β-glucanase to reduce the detrimental effects of a high viscosity feed was also observed. The study further showed that starch digestion was significantly higher in enzyme supplemented diets.

2.3.2 Arabinoxylan

Rye and wheat contain arabinoxylans which are composed of polysaccharides having a backbone of xylose (1,4-linked) with side chains of

arabinose residues (1,3-linked). Arabinoxylans are water soluble and are responsible for sticky droppings and a reduction in bird growth (Hesselman and Aman, 1986). They also contribute to the high viscosity of rye and wheat (Choct, 2001). Arabinoxylans present at more than 2% in feed can decrease nutrient digestibility and absorption (Hesselman and Aman, 1986). Wheat arabinoxylans, which are mainly located in the endosperm and the bran, can affect digestion of starch, fat and protein and therefore, act similarly to β-glucan in barley. Bedford and Morgan (1996) showed significant improvements in growth performance and digestion in poultry when wheat and rye based diets were supplemented with arabinoxylanase. Improvements shown by using these enzymes were not a result of reducing the NSPs to simple sugars, ostensibly to be easily digested, but rather by hydrolyzing (debranching) the linkages liberating smaller polymers and, therefore, reducing the viscosity of the digesta. Apajalahti (1998) demonstrated that inclusion of xylanase (2500 μg /kg feed) in wheat-based diets fed to poultry resulted in consistent changes in the composition of caecal microflora. It should be pointed out, however, that xylanases can show a great variation in activity depending on the structure or degree of branching of the arabinoxylan since the latter are known to vary in structure and location, even within the same plant (Austin et al., 1999). Vahjen et al. (1998) using xylanase (0.24 g/kg) supplemented feed also reported changes in the microbial flora of broilers. Examining both the luminal contents and tissue of the small intestine, the researchers reported that lactobacilli numbers were higher in tissues from birds that were fed enzyme-treated feed. Also, during the first 3 weeks of the trial,

presumptive enterobacteria and total gram positive cocci in luminal and tissue samples were lower in broilers maintained on the enzyme-supplemented diet.

2.3.3 Non-starch polysaccharides of soybean meal and canola meal

Pectic polysaccharides are the major components of the cell wall matrix of soybean and canola. They are characterized by a linear chain of α-(1-4)-D-galacturonic acid units with varying amounts of α-(1-2)-L-rhamnosyl residues. The galacturonic backbone is interrupted with rhamnopyranosyl residues and substituted with sidechains consisting mainly of L-arabinose, D-galactose and D-xylose (Aspinall, 1980). The term pectin also includes certain neutral polysaccharides lacking the galacturonic acid backbone, such as arabinans, galactans, galactomannans and arabinogalactans. Other polysaccharides in soybean and canola include cellulose, xylans, arabinoxylans and xyloglucans, which are predominantly found in the hull fraction.

Guar gum is a hydrophilic polysaccharide obtained from the seeds of the plant *Cyamopsis tetragonoloba*. It is a galactomannan with a (1-4 linked) β-D-mannopyranose backbone and contains α-D-galactose side chains (Merck Index., 2004). Guar gum is used industrially as a thickener and a stabilizer; the effectiveness of both properties increases with the amount of galactose side chains present on the backbone. Guar gum, a component similar to galactomannans of soybean meal, is not hydrolyzed in the small intestine or the colon and therefore is excreted in the feces (Blaut, 2002). It can constitute up to 23% of the diet (Pettey

et al., 2002) and while its nutrient potential is often ignored it also contributes significantly to high viscosity digesta. An enzyme that could hydrolyze this feed component in order to reduce viscosity and allow for the resulting breakdown products to be used in the digestion process would be of benefit. Enzymes most often used to breakdown guar gum include galactanases which hydrolyze galactose side chains and reduce the stiffness of the molecule (Merck Index, 2004).

2.4 Use of Exogenous Enzymes

Exogenous enzyme supplementation provides more nutrients to the animal from the initial diet by breaking down those compounds that cannot be digested by the animal's own enzymes. In addition, exogenous enzymes have been shown to alter the absorption of fats, fatty acids and fat-soluble micronutrients (Bedford, 2000). By making feed utilization more efficient, less feed is required making it more cost effective. Further, less cost is associated with the disposal of waste products, including excreta. The first known use of enzyme addition in poultry feed was in 1925, but the use of enzymes to break down NSPs began in the 1980's and rose sharply after 1992 (Brufau *et al.*, 2001). Previously, exogenous enzymes were used in the feed industry to reduce the viscosity of NSP in cereals such as barley, wheat, and rye thereby improving digestibility. Many studies have shown the clear benefits of enzyme supplementation which have led to further developments in enzyme technology to improve specificity and increase stability (Brufau *et al.*, 2001). The use of enzymes, encouraged by the partial ban of

antibiotic use in animal feeds in Europe, may also benefit animal health. Bedford (2000) suggested that specific diet formulations including exogenous enzymes, that have a beneficial effect on gut microflora, could be a viable alternative to antibiotic supplementation. Further, he suggested that although a reduction in viscosity was the major action of exogenous enzyme addition, possible beneficial effects on gut microflora should not be overlooked. Apajalahti (1998) for example, reported that the caeca of broiler chickens fed wheat-based diets with added xylanase contained increased levels of *Peptostreptococcus*, *Bacteroides*, *Propionibacterium*, *Eubacterium* and *Bifidobacterium* but reduced levels of *Clostridia*, *Enterobacteriacea* and *Campylobacter*. Although exogenous enzymes have been shown to reduce viscosity, it remains unclear where in the gut these effects occur and exactly how gut bacteria are altered by enzyme addition (Philip *et al.*, 1995). Further, birds under 8 weeks old are known to have higher digesta viscosity which decreases with age; therefore the value of using exogenous enzymes in older birds is more difficult to assess (Chesson, 2001) especially since there is no direct method of measuring enzyme activity in the gut. Changes in intestinal viscosity, however, have been used as an indirect measurement (Philip et al., 1995).

Further, experimental variation in results can occur due to the amount of enzyme added to the feed. For example, Yasar and Forbes (2000) used 1 g of glucanase enzyme per kg of feed as recommended by the suppliers (Finnfeeds International). In contrast, Vahjen *et al.* (1998) used 0.24 g xylanase/kg of feed to determine effects on natural microflora of chicks. Zhang et al. (1996) used both xylanase and glucanase at 0.33, 1 and 3 g/kg in order to study variation in growth performance of leghorn chicks. The authors found that there were differences in growth performance using different amounts of

enzyme. They noted that growth performance increased with the higher levels of enzyme addition. However, the response to enzyme treatment was not directly proportional to the amount of enzyme added. It was also noted that growth differences were much more apparent in the first week of the experiment than in the second.

It is difficult to determine how well feed supplemented enzymes survive under varying conditions in the gut. Although *in vitro* studies have shown that these enzymes are sensitive to the pH in the small intestine, they are also known to be destroyed by endogenous enzymes (Philip *et al.*, 1995). Silva and Smithard (2002) showed that 15-20% of the original xylanase added to a rye-based diet remained active after passing through the upper gut in poultry. Therefore, it seems that some enzyme activity remains in the small intestine. In addition to enzyme activity, the method of application of the enzyme to feed is also important. Generally, enzymes cannot be added to feed prior to processing (pelleting) because high temperatures created (up to 95°C) would inactivate them. Thermolability of enzymes creates problems for feed manufacturers due to the extra costs involved for machinery and the possible need for feed mill expansion (Bedford, 2000). Further, commercially available enzymes are limited microbial products where microorganisms produce large quantities (Philip *et al.*, 1995). While it is desirable to use enzymes with high specific activity, it is not always possible to obtain them at the quantities needed as feed supplements. In the future, by using recombinant DNA technology it may be possible to produce enzymes in sufficient quantities (Philip *et al.*, 1995). Also, it is unclear whether a single or complex mixture of enzymes is necessary to produce optimum dietary digestibility. There is a need for the latter issue to be clarified to allow optimized feed utilization.

2.4.1 Predicting the response to enzyme supplementation

Various factors affect the beneficial effects of exogenous enzyme supplementation in feed. Some of these factors include the nature of the feed or components, whether the feed has been processed and whether the appropriate enzyme and or concentrations were used. As previously stated, NSPs contribute to decreased nutrient digestion and therefore by measuring the quantity of NSPs in a diet and adjusting the enzyme amount accordingly it may be possible to produce predictable results and determine the amount of enzyme required. However, problems arise in determining the types of NSP present since low-cost diets contain many plant ingredients with different forms of NSP making it impossible to know the amounts and types of NSP present by quantitative measurements (Choct, 2001). There is significant variation in the quantity and composition of NSPs not only among different cereals, but also within cereals and even within individual species (Marquardt, 1997). Further, the amount of viscous carbohydrates recovered from a cereal can vary due to method of isolation, variety, soil, climate, agronomic practices, degree of maturity at harvest and storage conditions. (Marquardt, 1997). In addition, the response of animals to enzyme-supplemented diets will depend on their age and wholesomeness of the diet. For example, older birds fed a high quality diet may not exhibit a significant change in growth compared to chicks fed a low quality feed (Acamovic, 2001).

Overall, however, there appears to be a correlation between the amount of NSPs in cereals and digesta viscosity (Marquardt, 1997). Therefore, measuring

gut viscosity could be used to assess the level of enzyme activity, however many factors must be considered such as the sections of intestine where sampling occurs and the age of the bird (Choct, 2001). Viscosity in different sections of the gut vary since nutrients are digested and absorbed as digesta moves down, causing indigestible portion (mainly NSP) to accumulate (Choct, 2001). Viscosity of the gut decreases as the bird ages allowing older birds to better digest diets containing ingredients such as barley (Choct, 2001). Choct and Kocher (2000) hypothesized that as a bird gets older, its microflora becomes more established and can better adapt to the environment such as producing small amounts of NSP-degrading enzymes to cope with viscous digesta. Therefore, gut viscosity values could be used to predict the response to enzyme supplementation by indicating the amount of NSP present; however measuring viscosity is a slow and expensive process (Choct, 2001). A promising method of measurement is the extract viscosity assay which involves using ground samples of feed ingredients that are extracted in water or buffer (Choct, 2001). Rotter *et al.* (1989) and Choct *et al.* (1993) have successfully used this indirect measurement for NSP.

2.4.2 Method of enzyme supplementation

The method of addition of exogenous enzymes to poultry feed can vary depending on the product and the producer. Enzymes can be added as a powder or as granules to the diet before mixing and pelleting (Acamovic, 2001). This allows better distribution of the enzyme throughout the feed and maximizes

contact with the target substrate. However, the majority of enzymes cannot withstand the high temperatures encountered in processing. It appears that steam used during the pelleting process is responsible for the loss of enzyme activity. A study by Inborr and Bedford (1994) indicated that glucanase could remain stable under dry heat at 90°C for 30 min, however, if moisture was added in the form of steam at 95°C for 15 min only 20% of the enzyme activity remained. Xylanase seems to be more sensitive to heat than glucanase although at temperatures above 100°C, all enzyme activity is greatly reduced (Vranjes *et al.*, 1994). In order to avoid the deleterious effects of heat, alternatives have been developed including adding enzyme in liquid form after pelleting. This protects enzymes from exposure to high temperatures; however, the liquid largely coats the surface of the pellet and is not accessible to the entire substrate (Acamovic, 2001). Although this method does not necessarily prevent the enzyme from contacting more substrate once digestion begins, it tends to limit the amount of "pre-ingestion action" (Acamovic, 2001).

2.5 Gut Microflora

Significant levels of bacteria are present in the chicken gut even one day after hatching. Eggs produced under commercial production conditions are disinfected prior to hatching, although some bacteria still remain on the shell. Handling newly hatched chicks by humans for sex determination and transportation also leads to some cross contamination (Vahjen *et al.*, 1998). Overall, in a commercial environment, the

microflora of the chicken gut would be expected to differ from chicks hatched non-commercially. In this respect, the lower bacterial content of commercially hatched birds could leave them more vulnerable to colonization by pathogenic organisms. Conditions during hatching, such as hygiene and the immediate environment, determine the content and quantity of bacteria in the gut (Schutte and Langhout, 1999). This information led to the development by Nurmi and Rantala (1973) of competitive exclusion. This involves introducing birds to non-pathogenic bacteria very early in life in order to colonize their digestive tracts, making it more difficult for pathogenic bacteria to proliferate. This theory later contributed to the application of probiotics in animal diets.

2.5.1 Viscosity and gut microflora

Although increased viscosity of digesta has been used as a basis to explain the anti-nutritive effects of certain cereals, it alone cannot be responsible for altering weight gain and nutrient digestibility in poultry (Vahjen *et al.*, 1998). As previously stated, the response of poultry to NSP may also be directly or indirectly related to changes the microflora of their GIT (Choct and Annison, 1992). Absorption of nutrients by the host seems to be accomplished by an interaction between the host and the intestinal microflora to share available resources. Thus, the use of exogenous enzymes appears to shift this equilibrium in favor of the host animal (Bedford and Apajalahti, 2001). It is difficult to define this relationship due to the complex interactions between microorganisms, feed and animals (Vahjen *et al.*, 1998). The high viscosity of the digesta due to NSPs

can decrease transit time and therefore allow for higher proliferation of gut bacteria (Choct and Annison, 1992). Studies such as those by Choct and Annison (1992) and Vranjes and Wenk (1995) have shown increased performance of birds on barley or rye diets with antibiotic supplementation. Furthermore, research using germ-free broilers has indicated that an increase in viscosity of intestinal contents did not produce any negative effects. From this work Schutte and Langhout (1999) concluded that gut content viscosity may not be relevant unless specific bacteria in the GIT of the bird are taken into consideration. Therefore, in order to further assess how exogenous enzymes affect animals, their effects on gut microflora must be considered.

2.5.2 Effect of exogenous enzymes on gut microflora

It remains unclear how enteric microorganisms react to enzyme supplementation. The general consensus is that since enzymes break down more complex molecules to simpler constituents, bacterial metabolism is facilitated thereby encouraging proliferation in the gut and perhaps interfering with the growth of other bacteria, thereby changing the quantity and composition of the microflora (Bedford and Apajalahti, 2001). However, a study by Choct and Annison (1992) showed that when a diet was supplemented with arabinoxylanase, arabinoxylans were not broken down to simple sugars, but rather the chain lengths or degree of polymerization (DP) of the original molecules were reduced, thereby

reducing viscosity. Therefore, it is likely that NSPs may not be broken down completely and may not account for changes in gut bacteria.

The ability of poultry to absorb nutrients during digestion is, in part, dependent on the composition of the microflora and level of bacteria in their GIT. These bacteria are dependent on nutrients derived from the diet; therefore changes in diet should affect microflora composition and in turn the digestive system (Bedford and Apajalahti, 2001). Since viscosity has been shown to change with enzyme addition, the rate of flow of digesta will alter access of bacteria to nutrients thereby influencing their rate of growth. When both enzymes and antibiotics are included in diets, the response to enzymes is greatly reduced suggesting that enzyme response is decreased when microflora growth is impaired (Bedford and Apajalahti, 2001). This indicates a relationship between microflora and efficiency of exogenous enzyme supplementation. In this respect, Apajalahti and Bedford (1999) demonstrated that coliform, lactic acid bacteria, and enterococci levels decreased with addition of xylanase in a wheat-based diet (Figure 1). The authors noted a 60% reduction in microbial numbers in the xylanase supplemented treatment. Francesch et al (1999) also noted that levels of *Clostridium perfringens* in the jejunum of broiler chickens varied with cereal type and that enzyme supplementation tended to reduce their levels.

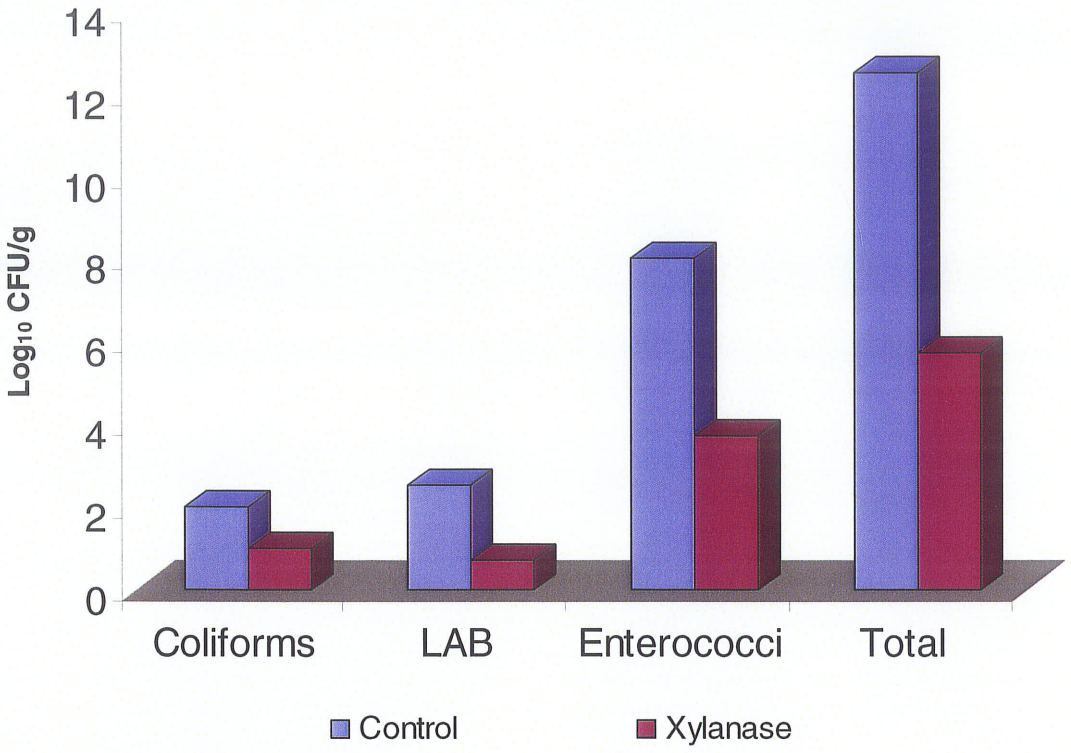


Figure 1. Influence of enzyme addition on total ileal microbial counts (Apajalahti and Bedford, 1999). LAB: lactic acid bacteria.

2.6 Probiotics

The increased interest in probiotics has been partially fuelled by the reduction of antibiotic use in animal feeds and by the need to find alternative methods to improve animal health and nutrition. There is concern that prophylactic use of antibiotics in feed will result in carry-over to consumer retail products and contribute to the development of drug-resistant bacteria in humans (Jin *et al.*, 1997). Although experimental results have shown mixed results regarding the efficacy of probiotics, the concept remains a point of interest. It is believed that by using probiotics to enhance the development of favorable

microflora, particularly in times of stress, the use of antibiotics can be reduced (Stavric and Kornegay, 1995). Probiotics, which consist of a live microbial feed supplement that improve the host intestinal microbial balance (Fuller, 1989), have been investigated for inclusion in feeds to improve animal health (Jin *et al.*, 1997).

The use of probiotics involves addition of live bacterial culture(s), usually lactic acid bacteria in high numbers ($>10^8$ colony forming units/ml) to the feed in order to replace or exclude pathogenic bacteria in the GIT, thereby improving bird performance and health. This process originally began with the theory of competitive exclusion (Nurmi and Rantala, 1973) where newly hatched chicks were given a live bacterial culture in order to establish a healthy intestinal tract, preventing colonization by pathogenic bacteria such as *Salmonella.*

2.6.1 Concept of probiotics

The GIT of broilers at hatching is nearly sterile and colonization by microflora begins immediately. The species and quantity of bacteria are dependent on the physiological conditions. Stringent hygienic rearing conditions can delay or disturb the creation of a healthy intestinal microflora (Stavric and Kornegay, 1995). Other factors that can delay colonization are antibiotic therapy and stress on the animal. The number of bacteria in the GIT ranges from about 10^9 to 10^{11} colony forming units per gram (cfu/g) of intestinal material (Stavric and Kornegay, 1995). The microflora can be classified into three different groups; the main flora, the satellite flora and the residual flora. The main group

makes up more than 90% and includes mostly obligate anaerobes and microaerophiles; bifidobacteria, lactobacilli and *Bacteroidaceae*. The satellite group, which contains less than 1% facultative anaerobes, contains primarily *Escherichia coli* and enterococci. The residual group (less than 0.01%) mainly contains *Clostridium, Proteus, Staphylococcus* and *Pseudomonas* species (Stavric and Kornegay, 1995). Studies of germ-free animals have shown that they are more susceptible to bacterial colonization by *Salmonella*, *Escherichia coli*, and *Clostridium*. Therefore, administering probiotics early in the life of poultry to colonize the GIT may help prevent establishment of pathogenic bacteria (Stavric and Kornegay, 1995).

2.6.2 Mode of action

The mode of action of probiotics remains speculative. There are many theories and some experimental evidence explaining how probiotics produce health benefits in animals and humans. Competitive exclusion, as previously described, is one of the most developed and researched modes of action. Nurmi and Rantala (1973) demonstrated the increased resistance of young chicks to *Salmonella* infection following inoculation with an adult bird fecal culture. They inoculated 1-2 day old chicks with a 1:10 dilution of a broth culture of adult poultry feces and monitored the numbers of *Salmonella* Infantis. Results showed that 100% of control birds were infected compared to 33% of treated birds (Jin *et al.*, 1997). This was the first of many studies designed to determine the efficiency

of probiotics. Most *in vitro* studies showed similar results; however, many field or *in vivo* studies have shown variable results. The precise mode of action of probiotics is not clear. Microflora could be competing with pathogenic bacteria for receptor sites on the intestinal mucosa and for nutrients (Stavric and Kornegay, 1995). It is possible that the microflora binds to the intestinal mucosa and prevents attachment of pathogenic bacteria at the same site or that binding of microflora crowds out the pathogenic bacteria from attaching in the same vicinity. Some probiotic strains such as *Lactobacillus* are known to produce antimicrobial agents such as bacteriocins, organic acids, and hydrogen peroxide (Jin *et al.*, 1997). These products, alone or perhaps more effectively, in combination, may change the conditions of the gut to impair growth of undesirable bacteria.

2.6.3 Probiotic concerns

The choice of a particular probiotic strain or combination of strains has been the subject of debate and study. Stavric and D'Aoust (1993) showed that undefined cultures from intestinal content were more effective than pure cultures. Nonetheless it is important that the identity of bacteria introduced to an animal be known to prevent introduction of pathogenic organisms during probiotic treatment (Jin *et al.*, 1997). It would be beneficial to select strains based on factors that would make them more effective such as resistance to gut conditions (pH and bile) and ability to adhere to gut mucosa. Further, results of some probiotic trials have shown such variability that skepticism over their true potential exists. This

variability could be due to use of different experimental conditions and protocols such as the age and health status of the bird and the nutritional adequacy of the diets (Jin *et al.*, 1997). The dose and strains of probiotics used could also affect the results. There are no regulations in place for strain selection and no standardized test protocols exist to determine efficiency. This is mainly due to the extreme variability in responses for different animals and ages. Further, there remains uncertainty as to the exact mode of probiotic action. Future problems to address include the development of probiotic preparations that remain viable through storage and strains that are specific for the animal and its environment (Jin *et al.*, 1997). Due to many variables in probiotic research there remains uncertainty and gaps of information that must be filled in order for this method to become universally accepted as an alternative to antibiotic use as growth promoters.

3.0 Enzyme supplementation in poultry diets: *in vitro* and *in vivo* effects on bacterial growth

3.1 ABSTRACT

The effect of exogenous enzyme supplementation on the growth of gut bacteria associated with poultry was determined *in vitro* and *in vivo*. The growth of fourteen bacteria commonly associated with the poultry gut were evaluated using four common feed substrates (β-glucan, xylan, guar gum, and raffinose) with or without addition of substrate specific commercial exogenous enzymes. While only *Bifidobacterium gallinarum* and *Campylobacter jejuni* grew on xylan with or without enzyme addition, nearly all bacteria grew on enzyme-supplemented raffinose except *Bifidobacterium pullorum* and *Lactobacillus acidophilus*. *Enterococcus gallinarum*, *Lactobacillus brevis*, *Bifidobacterium pullorum* and *Bifidobacterium animalis* grew on guar gum supplemented with enzyme. Selected microorganisms were also grown with or without enzyme supplementation (multi-carbohydrase enzyme) on soybean and canola meals which were previously subjected to ethanol extraction in order to remove simple sugars. None of the bacteria showed increased growth on soybean meal after 6 h with inclusion of enzyme except *C. jejuni*. With canola meal and enzyme supplementation only *C. jejuni, L. brevis* and *B. pullorum* showed increased growth. A chicken growth trial was also performed using wheat, barley and corn soybean-based diets with addition of exogenous enzymes and/or supplementation with a two species *Bacillus* probiotic. Target microbial groups were monitored in the caecum and ileum and included enterococci, *Escherichia coli*,

coliforms, aerobic and anaerobic spore-formers. In the wheat and corn-based diets, caecal contents of *E. coli* decreased with enzyme supplementation, but increased in the barley based diet. Caecal levels of both anaerobic and aerobic spore-forming bacteria typically increased with enzyme addition, however, in the ileum bacteriological changes were not apparent as a result of enzyme supplementation. The addition of probiotics had no effect on bacterial growth levels beyond that attained with enzyme supplementation. Poultry fed diets supplemented with exogenous enzymes had significantly higher weight gains and lower feed to gain ratios than poultry fed the control diet. Addition of probiotics did not significantly affect the growth performance of broilers.

3.2 INTRODUCTION

The use of antibiotic supplementation in animal feeds has been shown to be a factor contributing to the increase in antibiotic resistant bacteria in the human food chain and has resulted in a partial ban of antibiotic-based growth promoters for use in animal feed (Jin *et al.*, 1997). This has increased the need to find alternative methods to improve animal health and performance. Exogenous enzymes have been added to poultry feed for decades in order to reduce the viscosity of cereals high in non-starch polysaccharides (NSPs). Cereals such as wheat, rye, and barley contain NSPs that increase viscosity of the digesta, reducing the extent of digestion and quantity of nutrients absorbed (Sheppy, 2001). The purpose of supplementing diets with exogenous enzymes is to provide more nutrients to the animal from the feed by breaking down or depolymerizing those portions that cannot be digested by the animal's own enzymes. This could decrease the amount or

quality of feed required and increase profits to the producers since the feed component usually comprises the highest cost of production (Sheppy, 2001). Recent research has reported on the effect of exogenous enzyme supplementation on the gut microflora of poultry as well as its effect on common pathogenic microorganisms (Vahjen et al., 1998; Bedford and Apajalahti, 2001). Although the primary reason for exogenous enzyme addition is to reduce viscosity of feed, it has been suggested that intestinal microflora including pathogens could also be affected. Increased viscosity of digesta in commercial poultry has been shown to influence gut microflora in a negative manner (Choct, 2001). In addition, recent research has shown that exogenous enzymes may aid bird performance mostly by altering their gut microflora (Bedford, 2001). The composition and quantity of microflora are dependent, in large part, on diet. Changes in diet formulation can alter bacterial microflora which can influence feed digestion and the ability to absorb nutrients (Bedford and Apajalahti, 2001). A study by Schutte and Langhout (1999), using germ-free birds, concluded that feed viscosity alone may not directly affect bird growth and that the natural gut microflora of the bird must be taken into consideration. The interaction of exogenous enzymes with feed components and the resulting effects on enteric bacteria is complex and requires additional study. Exogenous enzymes hydrolyze or depolymerize feed components and many create substrates for microbial growth. It would seem that since enzymes are designed to break down complex molecules, simpler carbohydrates would be created. These simpler carbohydrates would then be accessible as an energy source by certain gut bacteria, increasing their numbers and perhaps crowding out or eliminating others (Sheppy, 2001). Therefore, exogenous enzymes may

alter or shift not only the population, but also the microbial composition of the GIT resulting in changes to digestion.

Two of the most troublesome anti-nutritive compounds in feeds are β-glucans and arabinoxylans which can be found in barley, wheat and rye (Choct, 2001). β-glucans are the most abundant polysaccharide in feed and many microorganisms can produce glucanases which can break them down. Arabinoxylans can be extracted from wheat flour and bran and contain xylan and arabinose. Due to various complex linkages, their hydrolysis requires a combination of enzymes that could also affect the growth of gut microflora via the liberation of gluco-oligosaccharides (Choct, 2001). Further exploration of the effect of exogenous enzymes on gut microflora is necessary in order to better understand how they may improve digestion and the overall health of the animal.

Along with exogenous enzymes, another feed supplement which could hold promise are probiotics. Due to the partial ban of antibiotic supplementation in some European countries (Bedford and Apajalahti, 2001), the need to find alternative methods to maintain animal health is increasing. Probiotics are live microbial feed supplements that can improve gut microflora and aid in the prevention of disease (Stavric and Kornegay, 1995). A recent review of probiotic use in poultry was given by Jin *et al.* (1997).

The objectives of this investigation were to determine the effects of exogenous enzyme supplementation on the growth of selected microorganisms using individual feed components and ethanol-extracted meals. An *in vivo* feeding trial to determine the effects of exogenous enzyme supplementation and probiotic addition on the growth of selected microbial populations in the caecum and ileum of poultry was also performed.

3.3 METHODS AND MATERIALS

3.3.1 Cultures, maintenance and inoculum standardization

Fourteen microorganisms were selected for investigation based on their presence as part of the enteric microflora of poultry broilers. *Enterococcus faecalis* (ATCC 7080), *Enterococcus gallinarum* (ATCC 49573), *Escherichia coli* (ATCC 13788), *Salmonella* Typhimurium (University of Manitoba Food Science Culture Collection, FSCC 98), *Salmonella* Pullorum (FSCC LHO S-325) and *Listeria monocytogenes* (FSCC 6LMB) were grown on tryptic soy agar (TSA; Difco, Becton Dickinson Microbiology Systems, Sparks, Md.) at 37°C for 24 h. *Clostridium perfringens* (ATCC 13124) was grown on Perfringens Agar base (OPSP, Oxoid Inc., Nepean, Ont.) containing supplements SR 76 and SR 7 (Oxoid) at 38°C for 24-48 h. *Lactobacillius brevis* (ATCC 14869), *Lactobacillus rhamnosis* (ATCC 7469), *Lactobacillus acidophilus* (ATCC 4356), *Bifidobacterium pullorum* (Health Canada Collection, HCC 320), *Bifidobacterium gallinarum* (HCC 319) and *Bifidobacterium animalis* (HCC 331) were cultivated using MRS Agar (Difco) in anaerobic jars containing gas generating kits (BBL GasPak Plus^{TM}); 24-48 h; 38°C. *Campylobacter jejuni* (HCC SH26) was grown on TSA (Difco) at 40°C for 24-48 h in jars containing gas generating kits (BBL Campypak Plus). All microorganisms were maintained in cryovials at -70°C in TSB plus 20% (v/v) glycerol for long term storage.

A single colony of each bacterium was transferred to 100 ml trypticase soy broth (Difco; TSB) and grown for 24-48 h using conditions previously outlined. Each culture was centrifuged at 6,500 x g for 10 min and pellets were washed twice in sterile saline (0.85%) and standardized to approximately10^8 cfu/ml with sterile saline (0.85%) using an Ultrospec 2000 (Pharmacia Biotech Inc., Baie d'Urfe, QC) 600 nm = 0.70-0.75.

3.3.2 Bacterial utilization of monosaccharides

All bacteria were initially tested for their ability to grow in glucose, fructose, mannose, galactose, xylose and arabinose (Sigma Chemical Co., St. Louis, Mo.) as a single source of carbohydrate. These monosaccharides represent the component sugars of the NSPs used in this study. It was of interest to determine the ability of microorganisms to utilize these sugars should they be released via enzyme hydrolysis. A basal medium was prepared by combining 15 ml sodium phosphate buffer (0.1M; pH 6.0) and 15 ml mineral salts solution (tryptone, 1.0 %, Difco; NaCl, 0.5%, wt/vol) (Koneman, 1988) in 100 ml flasks and sterilized (121°C, 15 min). Following cooling to room temperature, each filter sterilized (0.45 μm) monosaccharide was added (0.5%, w/v), the media were inoculated (1 ml of 10^8 cfu/ml) and incubated at 37°C. Aliquots (0.2 ml) from each flask were transferred to sterile, 96-well microtiter plates (Corning Inc.; Corning, N.Y.) and growth was monitored by recording the increases in absorbance at 340 nm using a Titertek Multiskan MCC/340 (Corning Inc.;

Corning, N.Y.). Lactic acid bacteria (LAB), *C. jejuni* and *Cl. perfringens* were monitored after 24 h due to their relative slower growth rates while all remaining microorganisms were monitored after 5 h. The net absorbance at each sampling time was determined by subtracting the control value (inoculated basal medium minus monosaccharide) from the measured value. Values presented are means from duplicate trials. An arbitrary change in absorbance of at least 0.05 at the end of 5 or 24 h was taken as a positive result.

3.3.3 Bacterial utilization of enzyme and non-enzyme treated oligosaccharide and NSP substrates

The substrates and enzymes used in this study are shown in Table 2 and were supplied by Dr. B. Slominski, Department of Animal Science, University of Manitoba. Each substrate (0.2 g) was boiled in 100 ml sodium phosphate buffer (0.1M; pH 6.0) for at least 5 min. Substrate solution (1.0 ml) was plated on TSA (Difco) and incubated for 24 h at 37°C to examine for contamination. Substrate solution (15 ml) was aseptically transferred into each of 6 sterile 125 ml Erlenmeyer flasks. Corresponding enzyme (0.003 g) was added to three of the flasks resulting in a substrate: enzyme ratio of 10:1. The remaining flasks were used as controls. The final number of bacteria in each flask was approximately 3 x 10^7 cfu/ml. Sterile basal media (15 ml) and standardized inocula (1.0 ml; 10^8 cfu/ml) were added to all flasks and incubated at 40°C to simulate the internal temperature of poultry and incubated for 18 h.

Growth was measured by monitoring the increase in absorbance at 340 nm using 0.2 ml aliquots from each flask transferred into 96-well microtiter plates. Absorbance measurements were made at 2 h intervals for 6 h and at 24 h of incubation using the Titertek Multiskan plate reader. For *B. pullorum*, *B. gallinarum* and *C. jejuni* absorbance was read at 24, 48 and 72 h due to their relative slow growth rate.

Table 2. Substrates and corresponding enzyme preparation.

Substrate	Enzyme	Commercial Enzyme Name[1]
β-glucan	β-glucanase A	BBG 10000
β -glucan	β-glucanase B	Biocellulase AZ
Xylan	Xylanase	Bioxylanase V
Guar Gum	Galactanase	Biogalactosidase 1000P
Raffinose	Galactosidase	Copan α-gal

[1] All enzymes provided by Canadian Bio-System Inc., Calgary, Alberta, Canada.

Each absorbance value recorded represents the means of triplicate trials.

Bacterial growth was also assessed by direct plating at 0, 6 and 24 h using the respective media as outlined previously. For *B. pullorum* and *B. gallinarum*, samples were incubated for 48 and 72 h, respectively, due to their relative slow growth. Plate count results were transformed to $\log_{10}$ values. A $\log_{10}$ increase ≥ 0.5 was arbitrarily chosen as a positive result. Values for direct plate counts represent means of duplicate trials. Serial dilutions were carried out using 0.1 % sterile peptone (Difco).

3.3.4 Enzyme activities and production of free sugars following incubation of pure polysaccharides with the corresponding enzyme preparations

All enzymes used in this study were provided by Canadian Bio-Systems Inc., Calgary, AB and the activities were determined by the supplier. Liberation of free sugars from the carbohydrate substrates by the action of commercial enzymes was determined using a Varian CP-3380 Gas Chromatograph. NSP was determined by gas-liquid chromatography (component neutral sugars) and by colorimetery (uronic acids). The procedure for neutral sugars was as described by Englyst and Cummings (1984) with some modifications (Slominski and Campbell, 1990). Uronic acids were determined using the procedure described by Scott (1979). Samples were evaluated as follows: each of the carbohydrate substrates (50 mg) was dissolved in 6 ml of sodium acetate buffer (0.1M; pH 5.2), boiled for approximately 1-2 min and cooled to room temperature. Enzyme (1 ml) was added to each carbohydrate-buffer solution and incubated for 16 h at 40°C. Myoinositol (5 ml; 1mg/ml prepared in saturated benzoic acid; Sigma Chemical Co.) was added and mixed thoroughly. Aliquots (1 ml) were combined with 5 µl of 2-octanol (Sigma Chemical Co.) and 0.1 ml of $NaBH_4$ (Sigma Chemical Co.), incubated at 40°C for 1h and reacted with 0.1 ml concentrated acetic acid. Part of the resulting solution (0.2 ml) was combined with 0.2 ml of 1-methylimidazole (Sigma Chemical Co.) and 0.2 ml of concentrated acetic anhydride and incubated for 30 min at room temperature. Five ml of water and 1

ml of dimethyl chloride (CH_2Cl_2) were then added with shaking and the final solutions were transferred to GLC vials for component sugar determination.

3.3.5 Bacterial growth in ethanol-extracted soybean and canola meals with and without enzyme supplementation

Canola and soybean meals were obtained from a local feed supplier. Each meal (6 g portions) ground to pass through a 1 mm sieve was combined with ethanol (40 ml, 80%) in 50 ml centrifuge tubes and shaken overnight in an environmentally controlled shaker at room temperature. The solution was centrifuged at 3000 x g for 10 min and the pellets extracted three more times with 80% ethanol as previously indicated. The final pellets were dried at 40°C.

Stock solutions of each meal were prepared by boiling 0.2 g substrate with 100 ml sodium phosphate buffer (0.1 M, pH 6.0) for 5 min and evaluated for contamination by plating on TSA (37°C; 24h). For each meal, a 15 ml boiled preparation was transferred to each of 6 sterile flasks. A multi-carbohydrase enzyme preparation described below was added to 3 flasks yielding a substrate:enzyme ratio of 10:1. The remaining flasks contained no enzyme and served as controls. The multi-carbohydrase mixture consisted of: xylanase (9,276 units/g), glucanase (11,306 units/g), cellulase (199 units/g), mannanase (2,214 units/g) and pectinase (2,000 units/g) (Meng *et al.*, 2005). All flasks were incubated for 18 h at 40°C following which sterile mineral salts medium (15 ml) and standardized bacterial inocula (1.0 ml; 10^8 cfu/ml) were added to all flasks

which were further incubated at 40°C. Bacterial growth was monitored by plate counts at 6 and 24 h using TSA (37°C; 24-48 h). Means were calculated based on triplicate trials each performed in duplicate. Serial dilutions were performed using 0.85% NaCl (w/v).

3.3.6 Broiler chicken experiment

3.3.6.1 Experimental diets

Three basal diets used in the study (Table 3) included wheat-, barley-, and corn-based diets each containing 20% of soybean meal (SBM) and 5-8% canola meal (CM). The diets were isoenergetic and isonitrogenous and were formulated to be lower in available energy and crude protein (95% of required amounts) than the NRC (1994) specification in order to make the birds more sensitive to enzyme effects. Other nutrients met or exceeded NRC specifications for broiler chickens. Each diet was mixed with or without appropriate enzyme supplement and probiotic (added at the expense of cereal components). Identification of experimental diets, the type of enzymes used, their inclusion rates and the characteristics of the probiotic product used are shown in Table 4.

Xylanase at 2000 units/kg diet and glucanase A at 700 units (u)/kg diet were added to a wheat/soybean/canola meal diet (treatments 2 and 3). Glucanase A or Glucanase B each at 2000 u/kg diet and xylanase at 700 u/kg diet were added to a barley/soybean/canola meal diet (treatments 5, 6 and 7). The enzyme

Table 3. Composition and calculated analysis of basal diets (%)

	Wheat/soybean/ canola meal diet	Barley/soybean/ canola meal diet	Corn/soybean/ canola meal diet
Ingredient			
Wheat	60.7		
Barley		54.6	
Corn			49.7
Soybean meal	20.0	20.0	20.0
Canola meal	5.0	5.0	8.0
Fish meal (64.2% CP)	5.1	8.6	2.6
Canola oil	5.3	8.5	5.5
Limestone[1]	1.4	1.3	1.3
Dicalcium phosphate[2]	0.95	0.45	1.35
DL-methionine	0.08	0.07	0.08
Mineral premix[3]	0.5	0.5	0.5
Vitamin premix[4]	1.0	1.0	1.0
Total	100.0	100.0	100.0
Calculated analysis			
Crude protein (%)[5]	22.0	22.0	22.0
AME (kcal/kg)[6]	3,050.0	3,050.0	3,050.0
Lysine (%)	1.1	1.1	1.1
Methionine (%)	0.5	0.5	0.5
Methionine + cystine (%)	0.9	0.9	0.9
Calcium (%)	1.0	1.0	1.0
Available phosphorous (%)	0.45	0.45	0.45

[1] Contained 38% calcium;
[2] Contained 21% calcium and 18% phosphorous;
[3] Mineral premix provided per kilogram of diet: Mn, 55 mg; Zn, 50 mg; Fe, 80 mg; Cu, 5 mg; Se, 0.1 mg; I, 0.36 mg; Na, 1.6 g;
[4] Vitamin premix provided per kilogram of diet: vitamin A, 8,250 IU; vitamin D_3, 1,000 IU; vitamin E, 11 IU; vitamin B_{12}, 0.012 mg; vitamin K, 1.1 mg; niacin, 53 mg; choline, 1,020 mg; folic acid, 0.75 mg; biotin, 0.25 mg; riboflavin, 5.5 mg;
[5] Calculated based on analyzed values;
[6] Apparent metabolizable energy.

preparation added to a corn/soybean/canola meal diet (treatments 9 and 10) was a multi-carbohydrase cocktail of cell wall degrading activities and provided 1000 U xylanase, 400 U glucanase, 1000 U pectinase, 120 U cellulase, 280 U mannanase and 180 U galactanase per kg of diet. Each enzyme-supplemented diet contained a probiotic mixture: Sporzyme BS923 composed of *Bacillus subtilis* 923 and Sporzyme BL21 containing *Bacillus licheniformis* 21 in a ratio of 4:1. The

probiotic was added to the diet at 0.01g/kg feed (treatments 3, 7, and 10). Enzyme supplements and probiotics were provided by Canadian Bio-Systems Inc., Calgary, AB, Canada.

Table 4. Identification of experimental diets used in the broiler chicken trial

Diet	Treatment
Wheat/soybean/canola meal	1. Control (no additive)
	2. Control + Xylanase[1]
	3. Control + Xylanase[1] + Probiotic[2]
Barley/soybean/canola meal	4. Control (no additive)
	5. Control + Glucanase A[3]
	6. Control + Glucanase B[4]
	7. Control + Glucanase B[4] + Probiotic[2]
Corn/soybean/canola meal	8. Control (no additive)
	9. Control + Multi-carbohydrase enzyme[5]
	10. Control + Multi-carbohydrase enzyme[5] + Probiotic[2]

[1] Provided 2000 U xylanase and 700 U glucanase per kilogram of diet.
[2] Combination of *B. subtilis* and *B. licheniformis*; included at 0.01g per kilogram of diet.
[3] Provided 2000 U glucanase and 700 U xylanase per kilogram of diet; regardless of enzyme activity, Glucanase A was found to be ineffective in producing free glucose from barley β-glucan.
[4] Provided 2000 U glucanase and 700 U xylanase per kilogram of diet; regardless of enzyme activity, Glucanase B was found to produce a significant amount of free glucose from barley β-glucan).
[5] Blend of carbohydrase enzymes used for soybean/canola meal cell wall polysaccharide depolymerization; provided 1000 U xylanase, 400 U glucanase, 1000 U pectinase, 120 U cellulase, 280 U mannanase and 180 U galactanase per kilogram of diet.

3.3.6.2 Bird management and sample collection

Four hundred one-day-old male Arbor Acres broiler chicks were obtained from a local commercial hatchery and housed at the University of Manitoba poultry barn. The birds were held in electrically heated Jamesway battery brooders (James

Mfg. Co., Mount Joy, PA) for a 4-d pre-experimental period and fed commercial chick starter crumbles (21% protein). On d 4, birds were fasted for 4 h, individually weighed and sorted into 5 weight classes. Groups of 5 birds were then randomly assigned to pens such that the average initial body weight of birds was similar across pens. Eight replicate pens of 5 birds each were randomly assigned to the 10 dietary treatments. All diets were fed in a mash form throughout the 2-wk experimental period. The birds had free access to water and feed and were provided with continuous light. Body weight and feed intake were monitored weekly using pens as the experimental units. Before weighing, the birds were fasted for 4 h. Mean body weight (BW), feed intake and feed-to-gain ratio were used to determine growth performance. All animal procedures were conducted according to the guidelines of the Canadian Council on Animal Care and the protocol for this study was approved by the Local Animal Care Committee of the University of Manitoba.

On day 18, 19 and 20, 18 birds from each treatment were randomly selected and killed by cervical dislocation. The contents of the ileum (from Meckel's diverticulum to 1cm above the ileo-cecal junction) and the caecum from 2 birds were collected and similar tissue pooled. Three pooled samples per treatment were obtained each day. This resulted in 30 samples a day for three days from each of the ileum and the caecum for a total of 180 samples. Each sample was maintained on ice for 2 to 4 h prior to processing.

Digesta samples were added to sterile peptone (Difco, 0.1 %) at a ratio of 1:100 in stomacher bags, massaged (Stomacher Lab-Blender 400, Seward

Medical, London, UK) for 1 min and further diluted using peptone. For *E. coli* and coliform determination, dilutions were plated on Petrifilm™ Coliform and *E. coli* plates (3M Canada Inc., London, ON) and incubated at 40°C for 48 h. Enterococci were determined by plating on Difco KF Streptococcal Agar with incubation at 37°C for 48 h. To determine spore-formers, dilutions were heated in a thermostatically controlled water bath at 80°C for 15 min in order to kill all non-heat resistant microorganisms. Samples were cooled in an ice bath and plated using a Spiral plater (Autoplate 4000, Spiral Systems Inc., Bethesda, MD) on TSA for aerobic spore-former determination; plates were incubated at 37°C and checked for growth at 24 and 48 h. For anaerobic growth, plates were incubated at 37°C in anaerobic jars with gas generating kits (BBL, GasPak Plus™) and growth was monitored at 24 and 48 h.

3.3.7 Statistical analysis

In order to analyze growth studies with ethanol extracted meals, all data was transformed to $\log_{10}$. Differences in growth with or without enzyme supplementation for each meal at each time period were compared for significance using the SAS analysis program with the standard t-test. A p-value of $P<0.05$ was considered statistically significant. For the broiler trial, all data were transformed to $\log_{10}$ and analyzed using the analysis of variance (ANOVA) procedure of SAS System 8e for Windows. Differences between means were compared using Tukey's test. A p-value of $P<0.05$ was considered statistically

significant. The body weight gain and feed-to-gain ratios were also compared using the ANOVA procedure of SAS System 8e for Windows.

3.4 RESULTS

3.4.1 Bacterial utilization of monosaccharides

Growth of pure cultures in minimal medium containing individual monosaccharides is shown in Table 5. Positive results were indicated by an increase in absorbance of at least 0.05 over 5 or 24 h. Based on this criterion, none of the microorganisms were able to utilize arabinose for growth. Xylose was predominantly utilized by *E. gallinarum* and *S.* Pullorum while glucose, as expected, was utilized by nearly all microorganisms with the exception of *B. gallinarum*, *L. acidophilus* and *C. jejuni*. Fructose appeared to have a beneficial effect on the growth of all bacteria tested with the exception of *B. gallinarum* and *L. acidophilus*. All microorganisms except *B. pullorum*, *B. gallinarum* and *C. jejuni* grew on mannose while only slightly half of the microorganisms grew on galactose. No apparent pattern in monosaccharide utilization was observed between pathogenic and probiotic/lactic acid bacteria. Also, as expected differences in growth due to carbohydrate utilization were observed between species and among genera.

Table 5. Bacterial growth (absorbance) in monosaccharides at 0.5%, w/v in basal medium. *

Bacterium	Absorbance (340 nm)[a]					
	glucose	fructose	xylose	arabinose	galactose	mannose
Listeria monocytogenes[b]	0.106	0.086	-0.056	-0.010	0.003	0.079
Enterococcus faecalis[b]	0.324	0.439	-0.044	-0.021	0.381	0.451
Enterococcus gallinarum[b]	0.273	0.341	0.251	-0.011	0.337	0.380
Escherichia coli[b]	0.285	0.193	0.053	-0.017	0.054	0.137
Salmonella Typhimurium[b]	0.288	0.264	-0.009	-0.005	0.118	0.206
Salmonella Pullorum[b]	0.214	0.206	0.186	-0.061	0.209	0.329
Clostridium perfringens[c]	0.317	0.361	-0.006	-0.014	0.039	0.292
Lactobacillus brevis[c]	0.416	0.443	0.003	-0.008	0.233	0.449
Bifidobacterium pullorum[c]	0.155	0.256	0.003	0.012	-0.015	-0.012
Bifidobacterium gallinarum[c]	-0.002	0.040	0.012	0.013	0.155	-0.032
Bifidobacterium animalis[c]	0.232	0.280	-0.051	-0.042	0.187	0.145
Lactobacillus rhamnosis[c]	0.164	0.174	0.015	-0.016	0.199	0.151
Lactobacillus acidophilus[c]	-0.054	-0.030	-0.029	0.000	-0.037	0.225
Campylobacter jejuni[c]	-0.015	0.599	0.071	-0.109	-0.024	0.026

[a] Represents net increase in absorbance (absorbance in mineral salts solution plus carbohydrate minus absorbance in mineral salts solution). Values are average of duplicates.
[b] Absorbance measured following 5 h of growth.
[c] Absorbance measured following 24 h of growth.
* All microorganisms were grown at 37-38°C with the exception of *C. jejuni* which was grown at 40°C.

3.4.2 Enzyme activity and production of free sugars following incubation of pure polysaccharides with the corresponding enzyme preparations

The activities of the enzyme preparations used in the current study are listed in Table 6. Both glucanases exhibited minor xylanase activity while the xylanase preparation also displayed some glucanase activity. All enzyme reactions resulted in free glucose, however, glucanase A and glucanase B yielded the lowest and highest levels, respectively (Table 7). Galactosidase yielded the highest amount of fructose, mannose and total free sugars while the galactanase preparation yielded the highest level of galactose. In addition, approximately 20% of the total free sugar liberated by the latter enzyme was glucose.

3.4.3 Bacterial utilization of enzyme and non-enzyme treated oligosaccharides and NSP substrates

Growth profiles of most bacteria in β-glucan supplemented with glucanase A (Figure 2) showed little or no difference when compared to growth in non-enzyme treated substrate. Nevertheless, based on the arbitrary criterion chosen (increase in absorbance of 0.05), only the following bacteria: *E. coli*, salmonellae and *L. acidophilus* did not exhibit a positive response as a result of enzyme supplementation. Overall, *E. gallinarum* exhibited the highest growth as

Table 6. Enzyme activities[1] of commercial glucanase A, glucanase B, xylanase, galactosidase and galactanase enzymes used in this study.

Enzyme	Enzyme Activity (units/g/min)			
	β-glucanase	xylanase	α-galactosidase	galactanase
Glucanase A *BBG 10,000*	9,084	126		
Glucanase B *Biocellulase AZ*	38,152	2,076		
Xylanase *Bioxylanase V*	2,273	91,237		
Galactosidase *Copan α-gal*			2,978	
Galactanase *Biogalactosidase 1000P*				1,000

[1] Canadian Bio-Systems Inc. Calgary, AB supplied enzymes and activity levels.

Table 7. Production of free sugars following incubation (16 h at 40°C) of β-glucan, arabinoxylan, guar gum and raffinose with corresponding commercial enzyme preparations.

Enzyme	Substrate	Free sugar released (mg/g)[1]					
		Arabinose	Xylose	Mannose	Galactose	Glucose	Total
Glucanase A *BBG 10,000*	β-glucan					2.2 ± 0.3	2.2 ± 0.3
Glucanase B *Biocellulase AZ*	β-glucan					859.3 ± 76.6	859.3 ± 76.6
Xylanase *Bioxylanase V*	Arabinoxylan	8.8 ± 0.4	96.9 ± 3.4			26.4 ± 2.1	132.1 ± 5.9
Galactosidase *Copan α-gal*	Raffinose			106.3 ± 0.4[2]	263.4 ± 7.1	479.6 ± 3.8[3]	849.2 ± 10.6
Galactanase *Biogalactosidase 1000P*	Guar Gum	3.9 ± 0.4		3.3 ± 0.4	270.1 ± 2.8	60.1 ± 2.5	337.3 ± 0.5

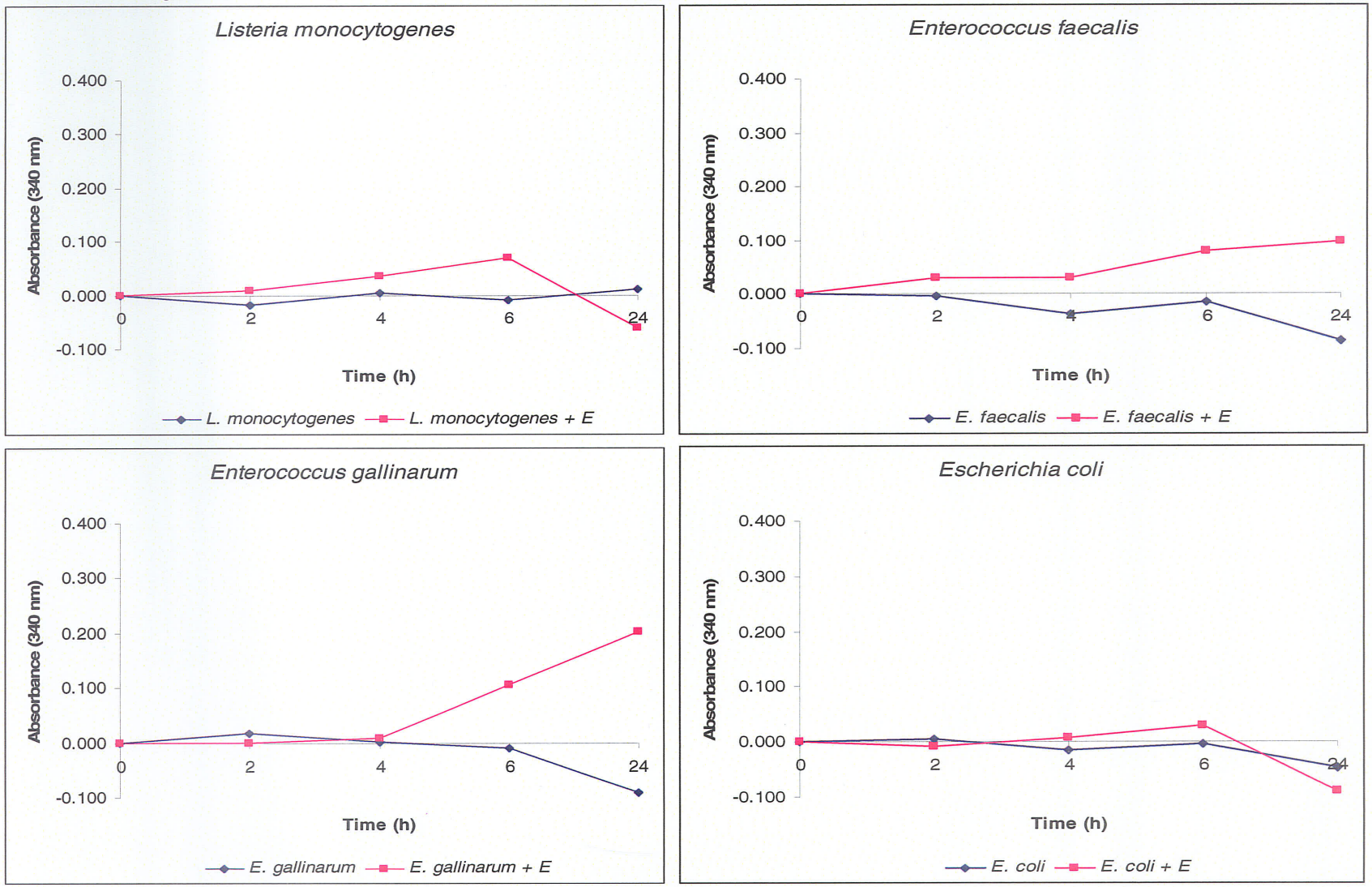
[1] As determined by GLC.
[2] Represents fructose rather than mannose. All results are averages of duplicate determinations ± standard deviation.
[3] Includes glucose and fructose.

a result of enzyme supplementation. The level and number of microorganisms growing on β-glucan containing glucanase B (Figure 3) appeared higher compared to glucanase A with the exception *L. acidophilus*. In contrast, most microorganisms did not show any growth enhancement on xylan containing xylanase (Figure 4). *S.* Typhimurium did show an increase but only at 24 h of incubation; *C. jejuni* and *B. gallinarum* also showed slight increases in growth after 24 h. Raffinose plus galactosidase (Figure 5) yielded an increase in growth for nearly all microorganisms with the exception of *L. acidophilus*, *C. jejuni* and *B. pullorum*. In addition, all pathogens exhibited an increase in growth as early as 4 h; however, some lactic acid bacteria required up to 24 h before differences in growth were observed.

All lactic acid bacteria, with the exception of *B. gallinarum*, showed an increase in growth when guar gum was supplemented with galactanase (Figure 6) Overall, only *Cl. perfringens* did not appear to benefit from the inclusion of galactanase.

Plate counts for similar trials are presented in Tables 8 to 12. When grown on β-glucan for 24 h, only *E. gallinarum*, *E. coli*, *Cl. perfringens* and *B. pullorum* exhibited increases in growth ≥ 0.5 log due to enzyme supplementation; *B. gallinarum* required 48 h (Table 8). Overall, no culture exhibited ≥ 1 log increase due to inclusion of glucanase A. When glucanase A was substituted by glucanase B (Table 9), the growth of many bacteria was greatly improved. *L. monocytogenes*, *E. gallinarum*, salmonellae, *L. brevis* and *B. pullorum* in

Figure 2. Bacterial growth in basal medium containing glucanase A (BBG 10,000) and non-enzyme treated β-glucan.



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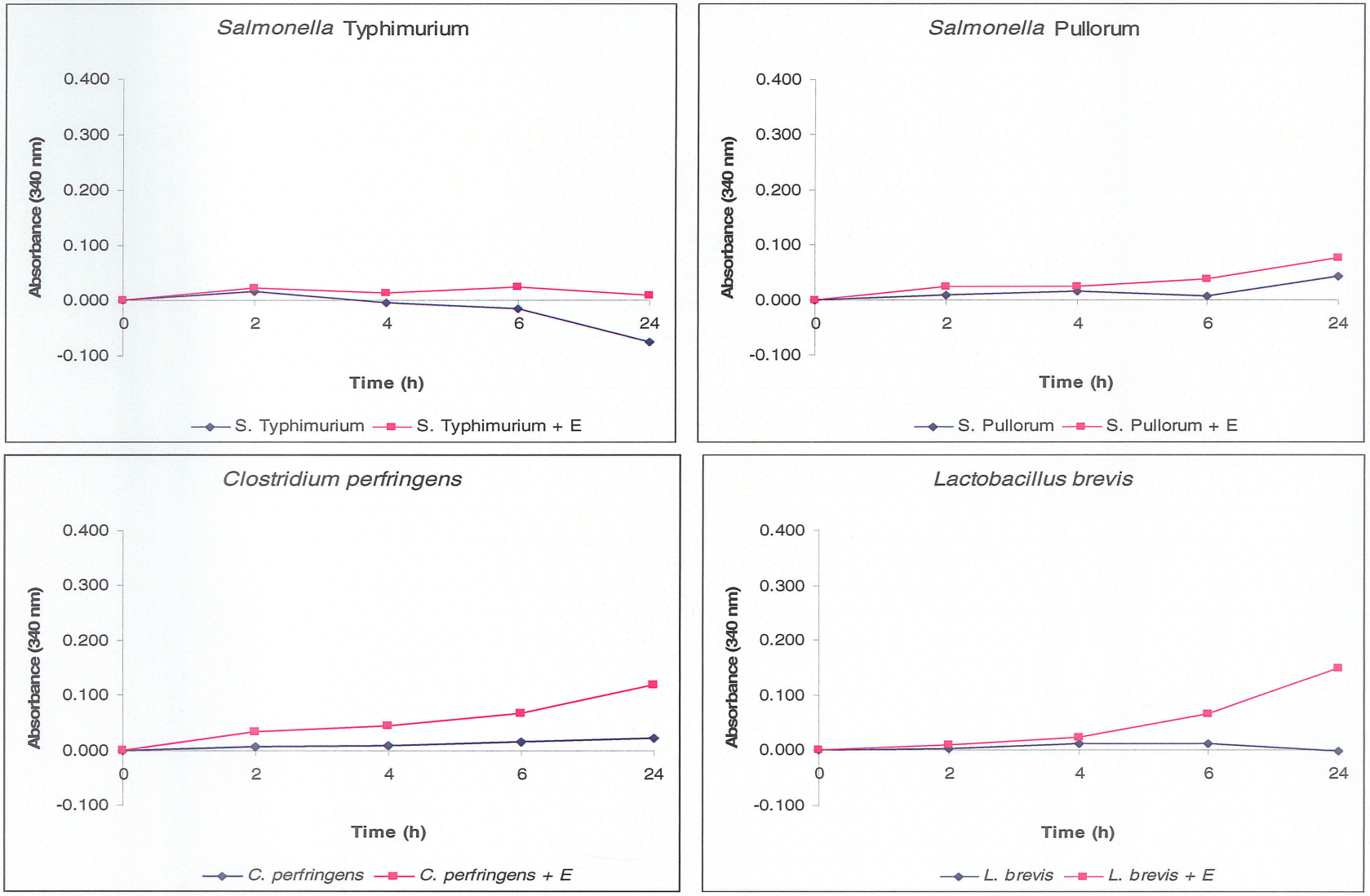

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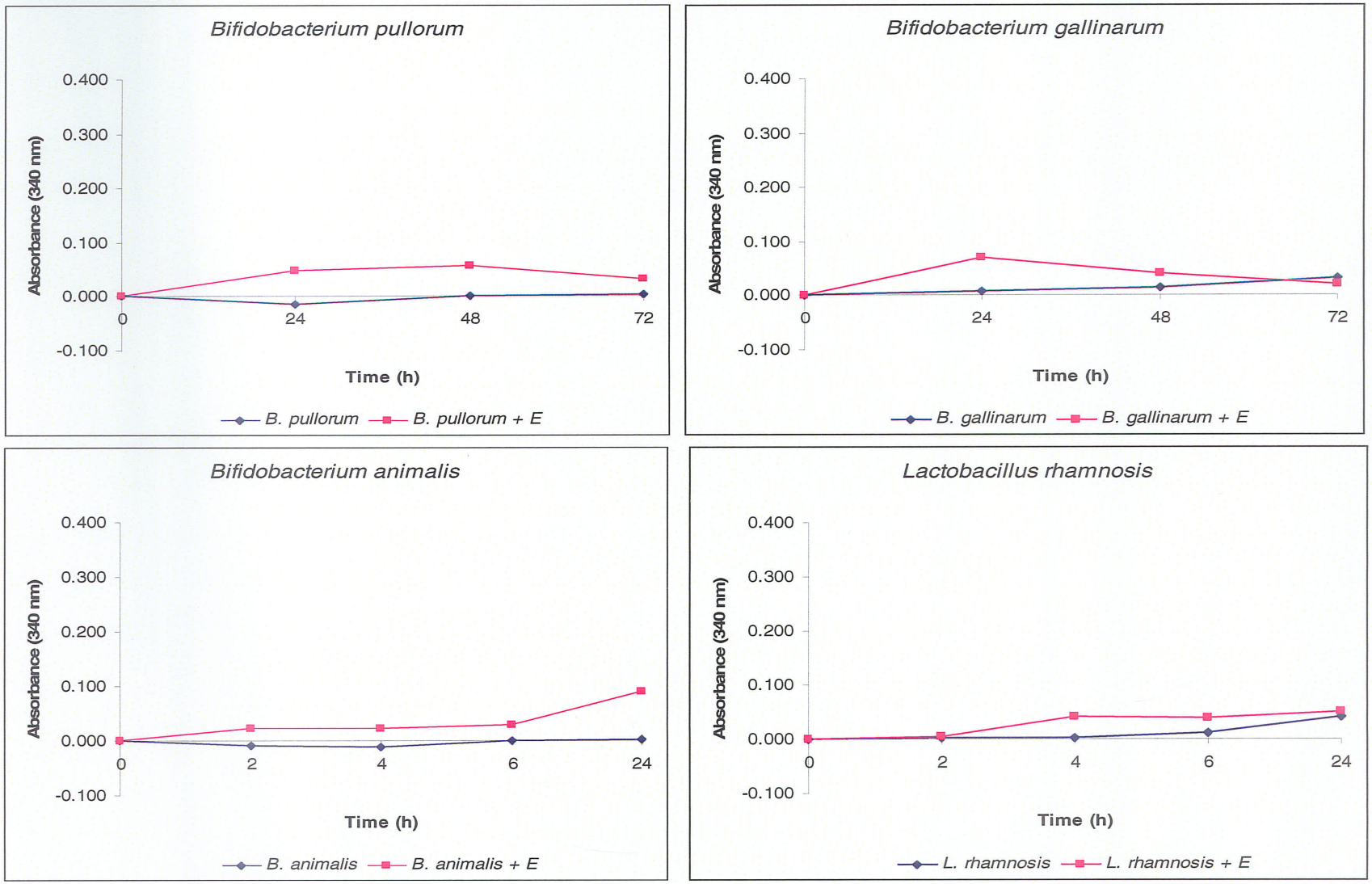

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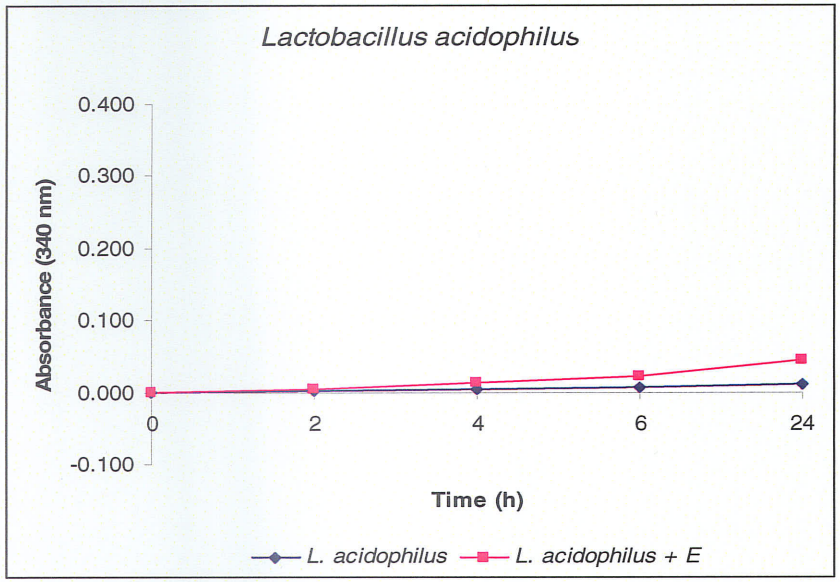


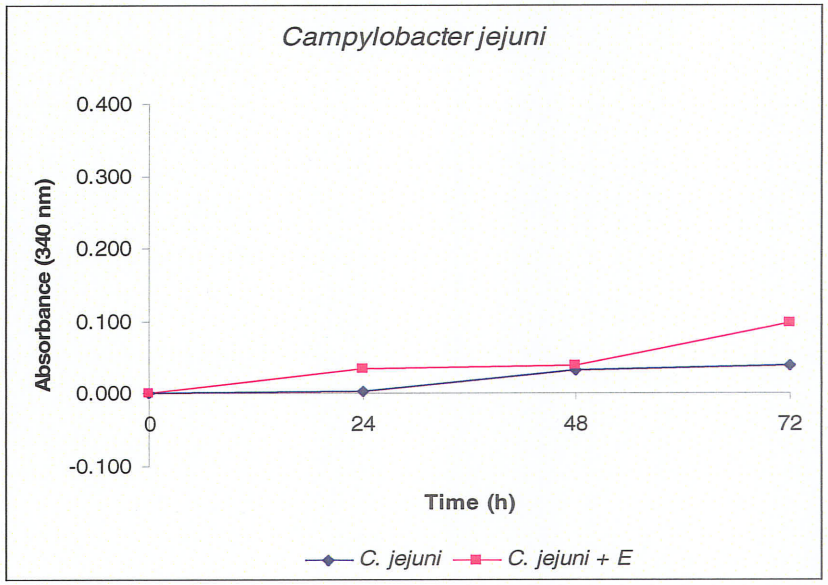
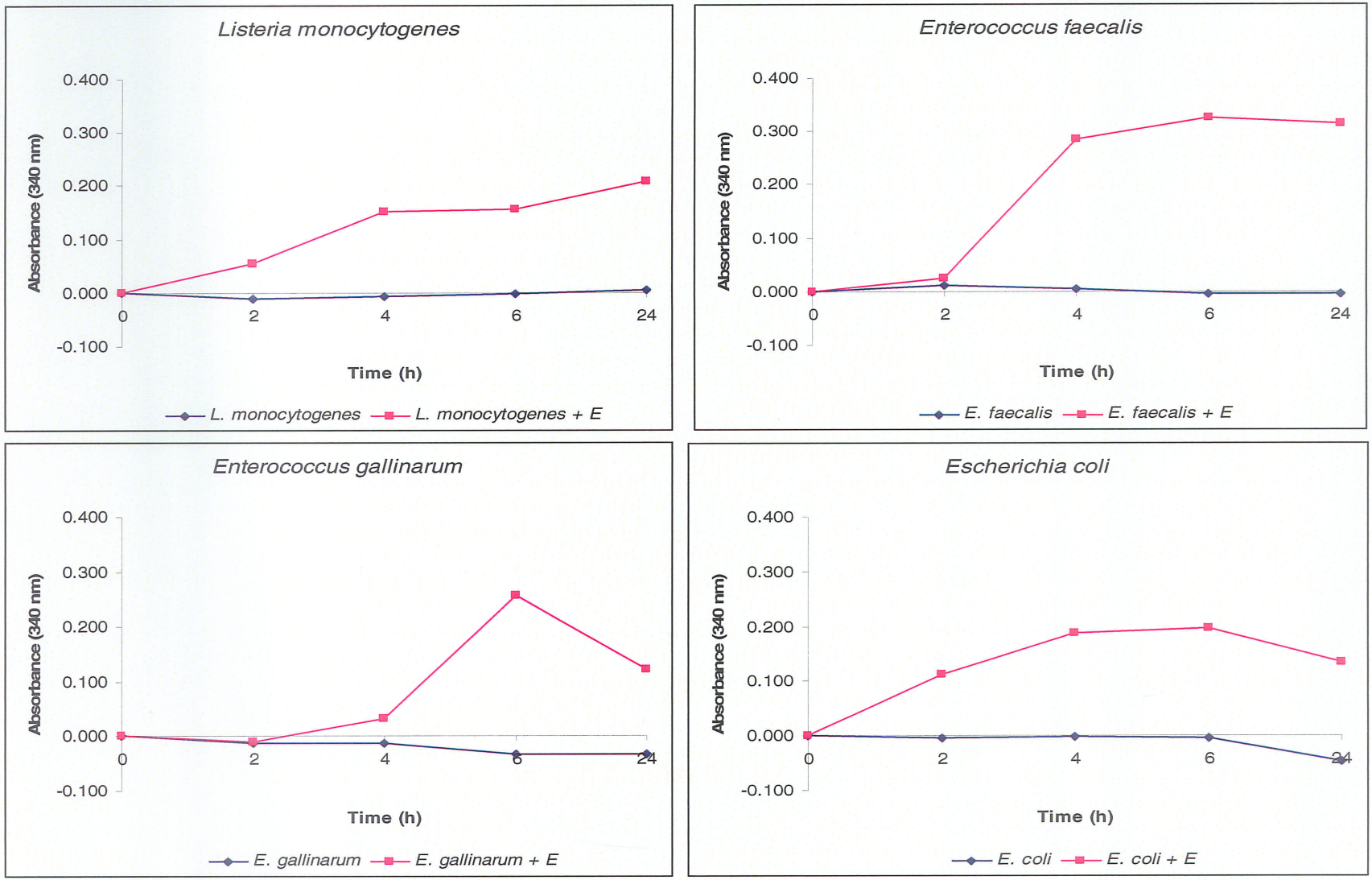


Figure 3. Bacterial growth in basal medium containing glucanase B (Biocellulase AZ) and non-enzyme treated β-glucan.



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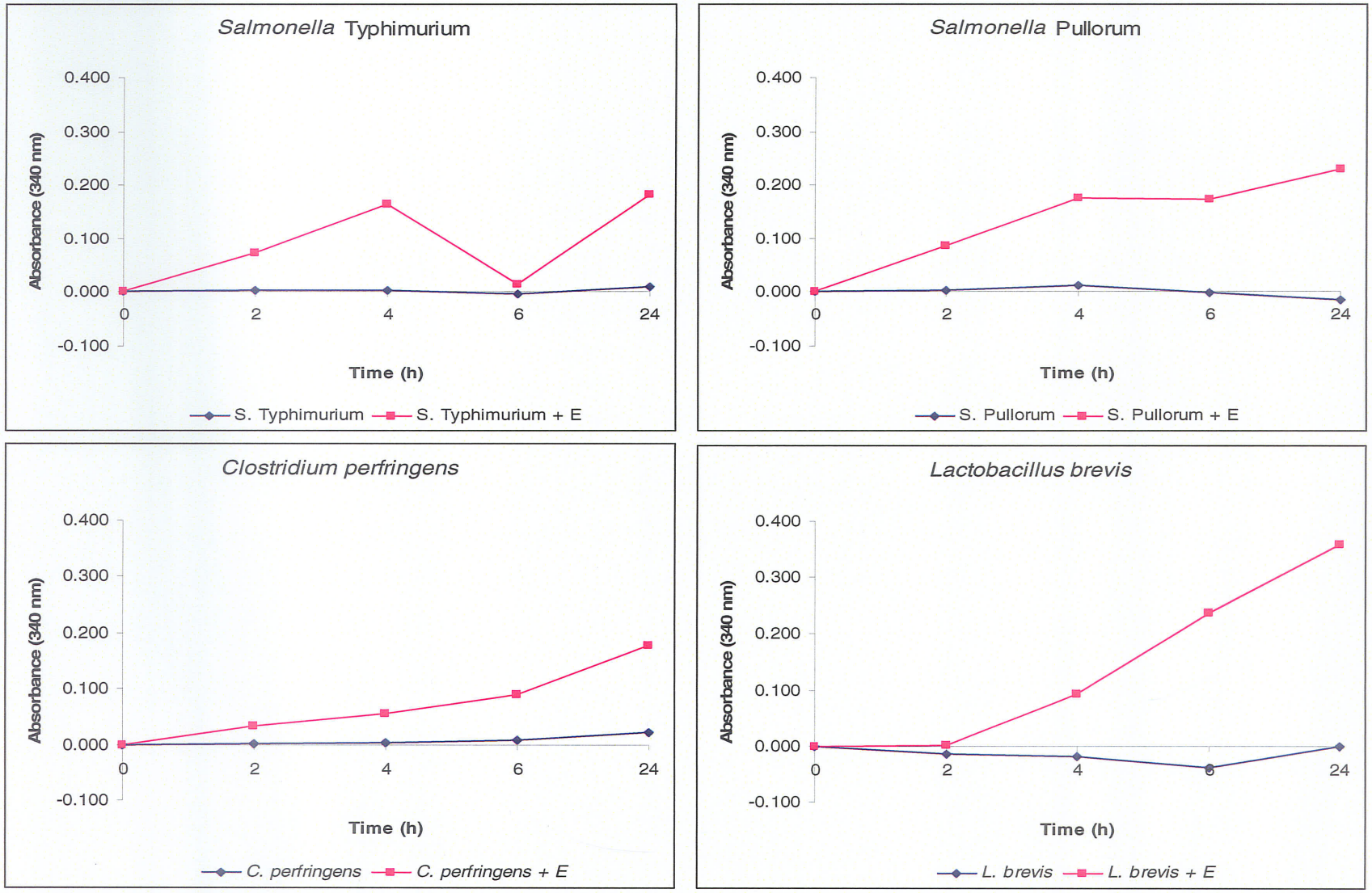

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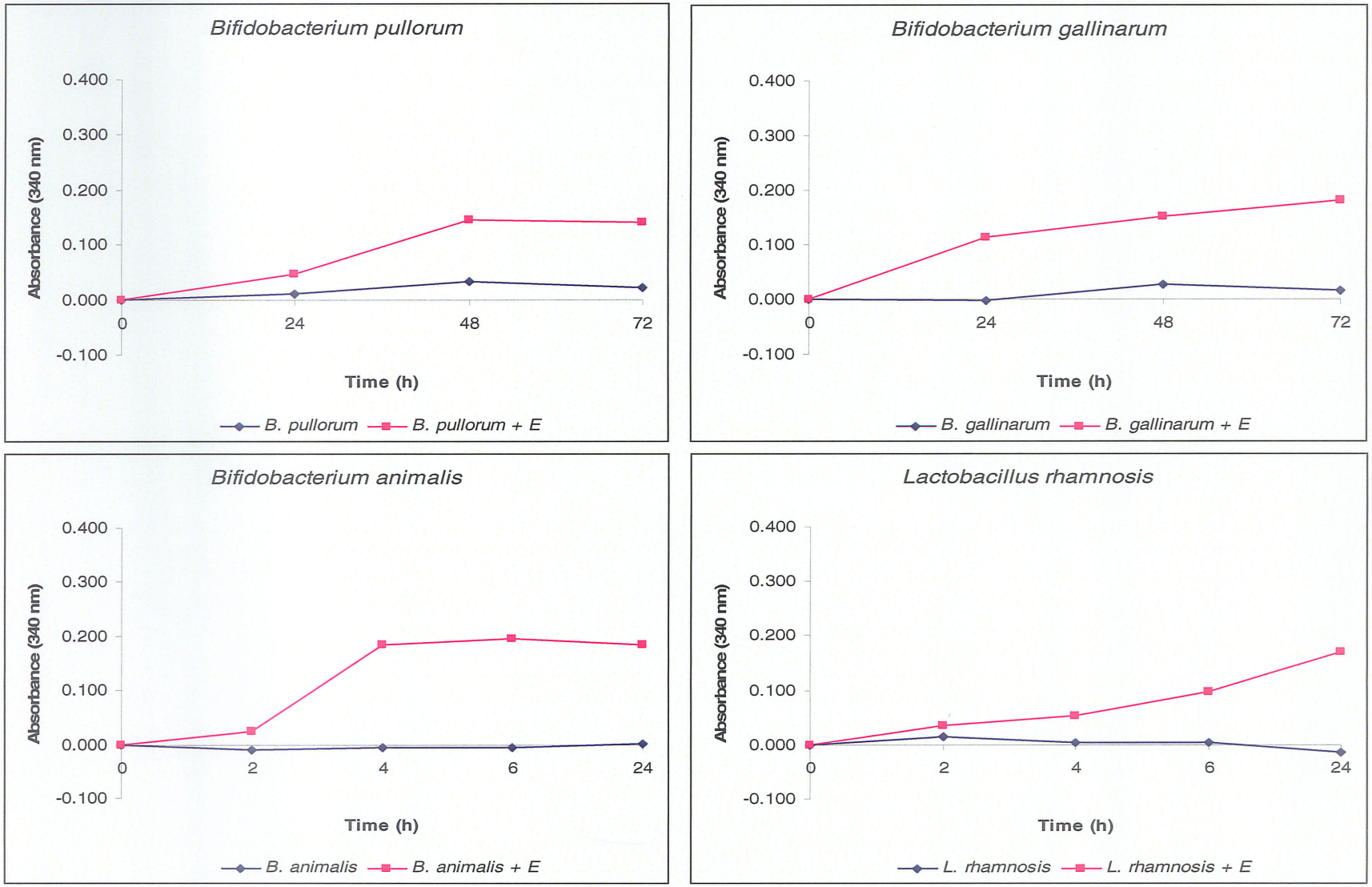

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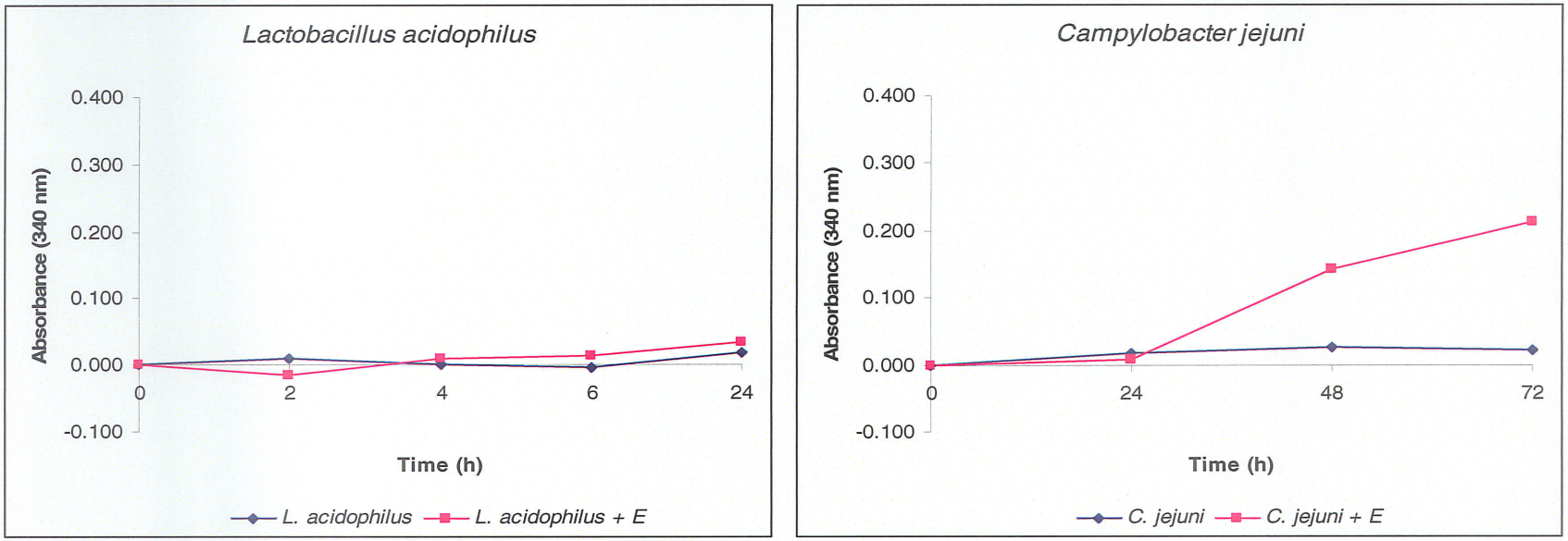
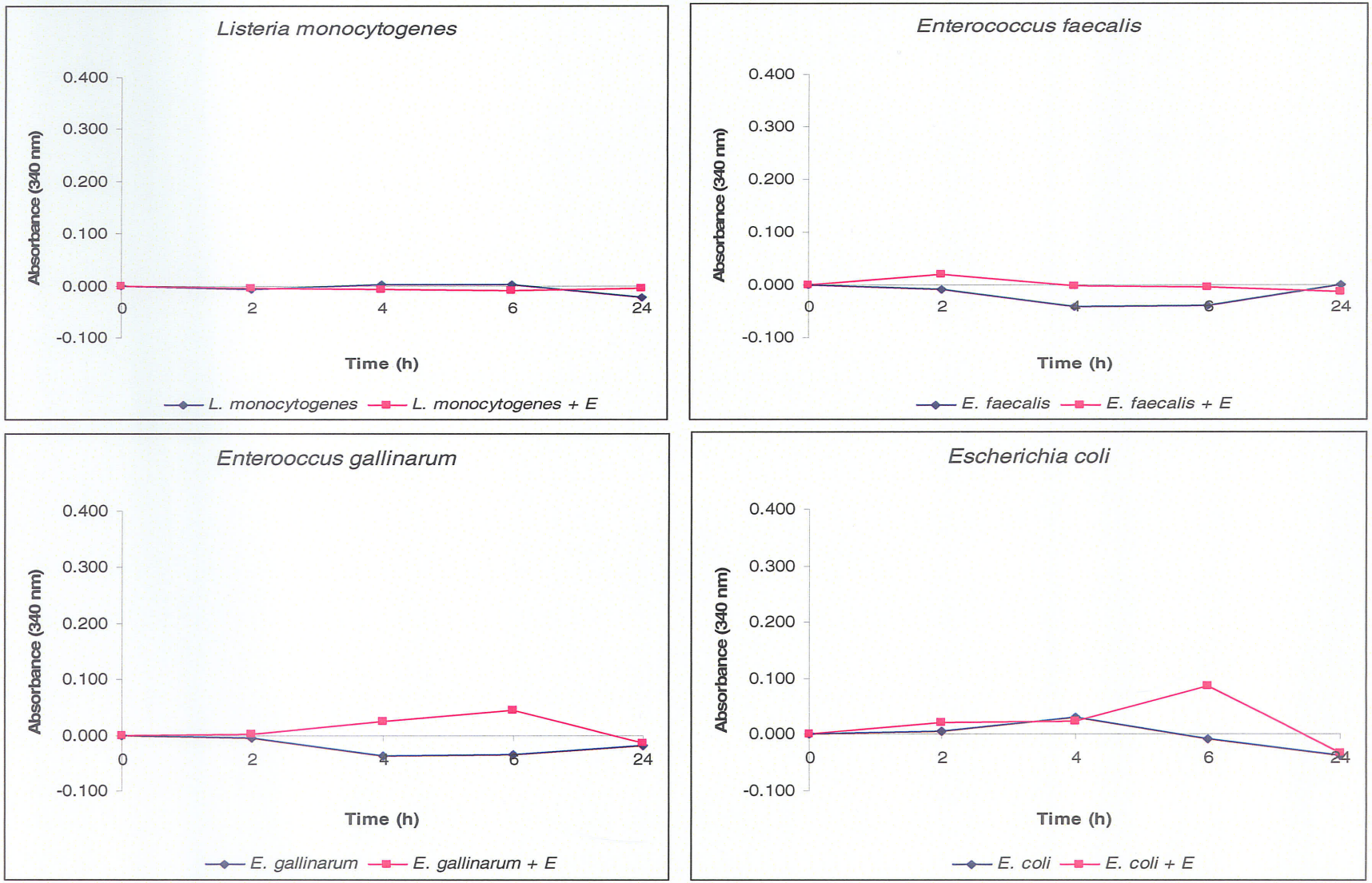


Figure 4. Bacterial growth in basal medium containing xylanase enzyme (Bioxylanase V) and non-enzyme treated xylan.



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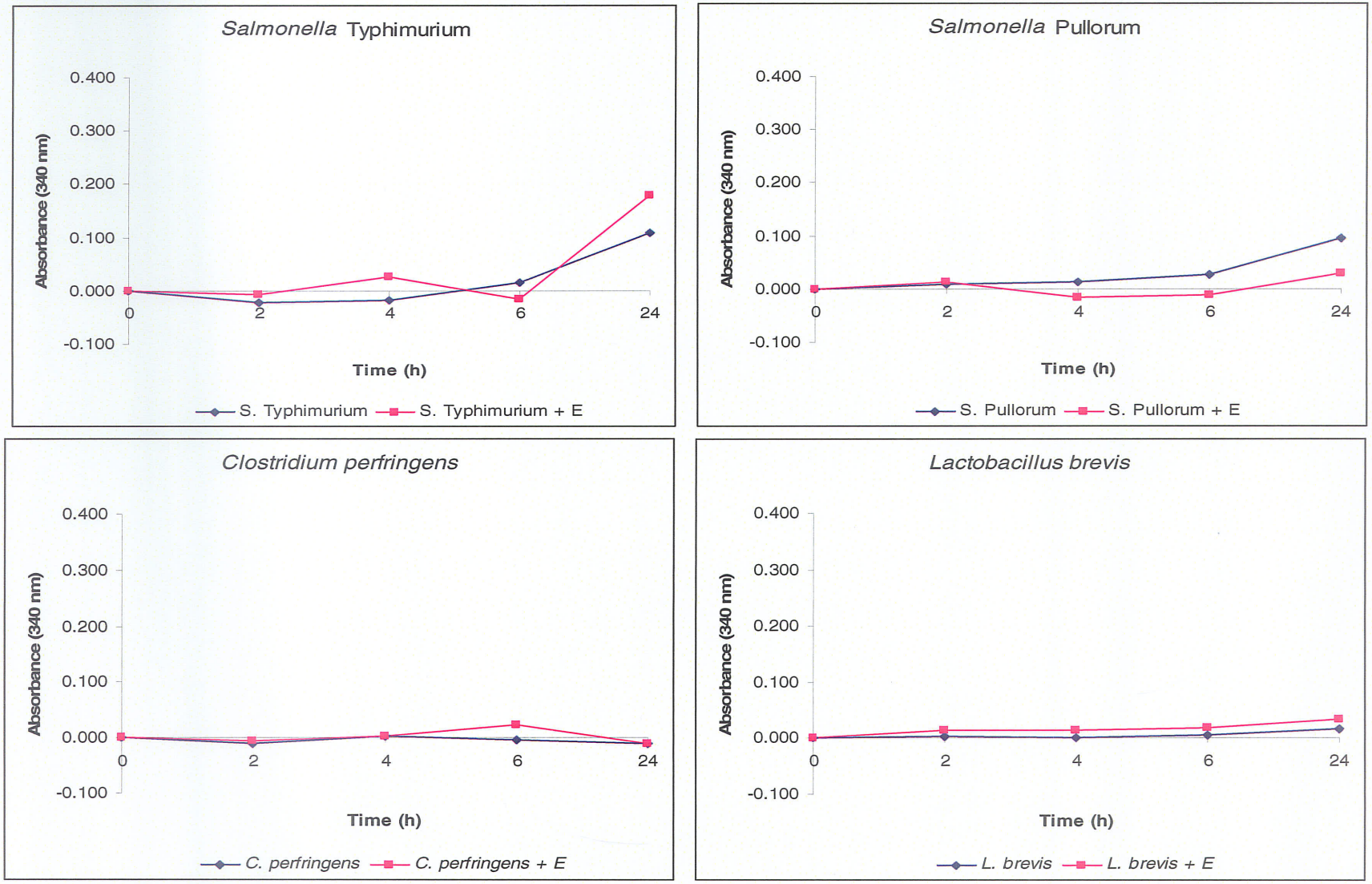

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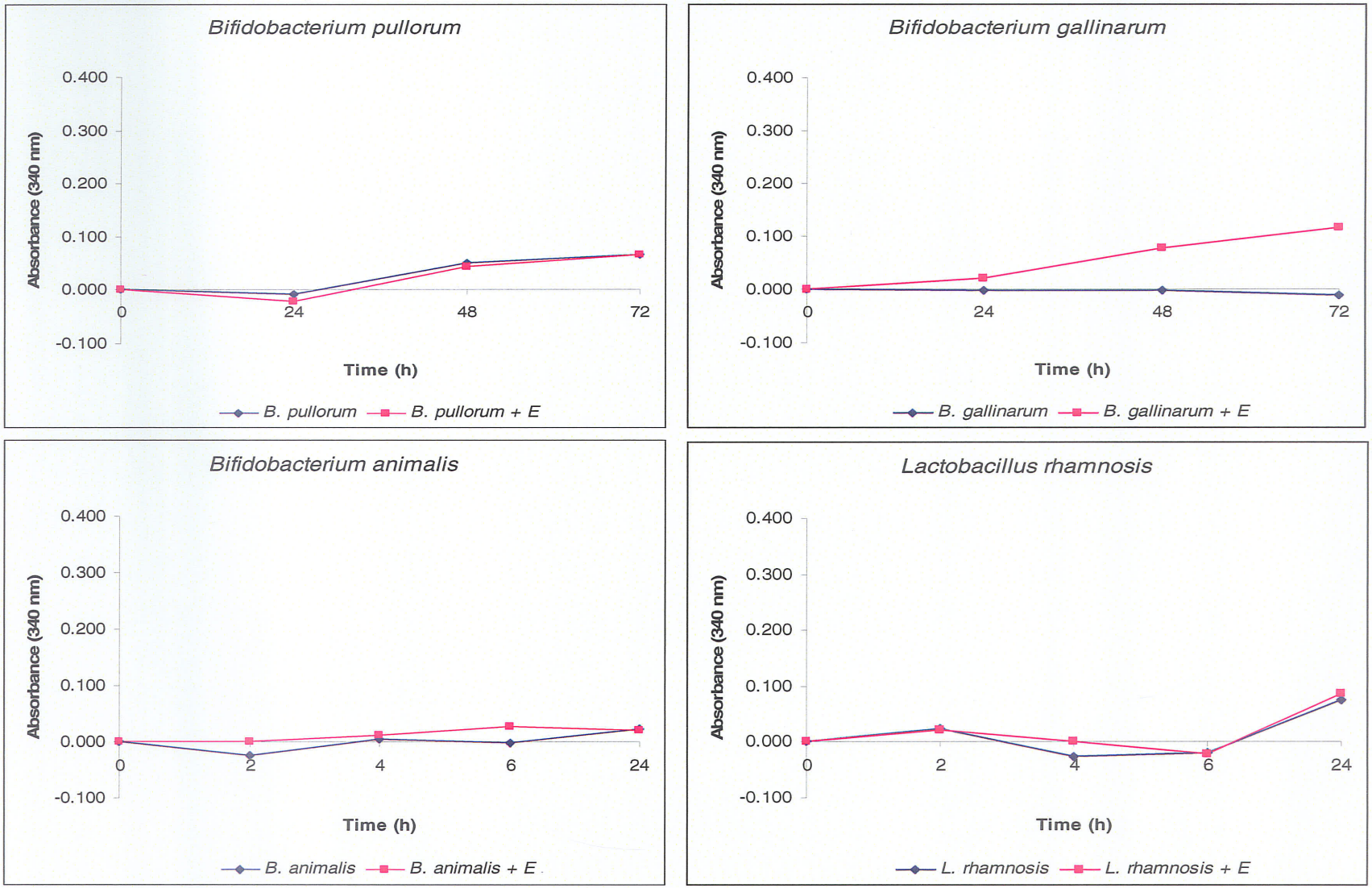

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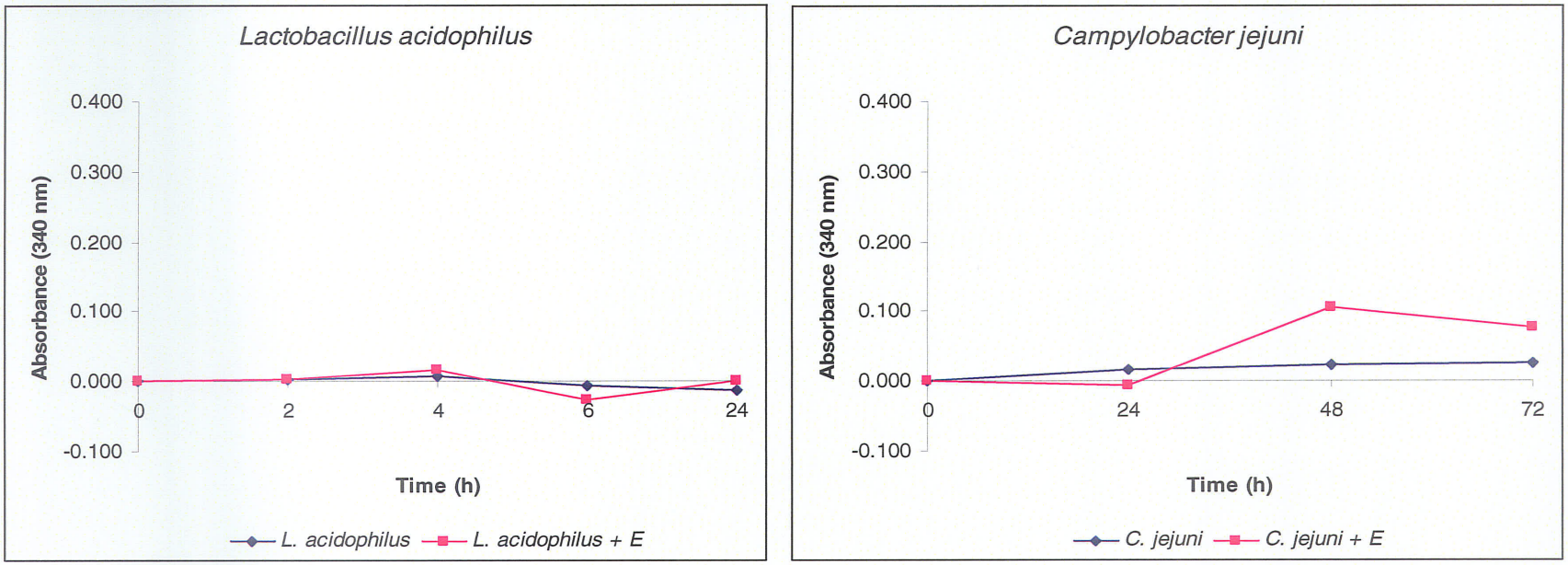
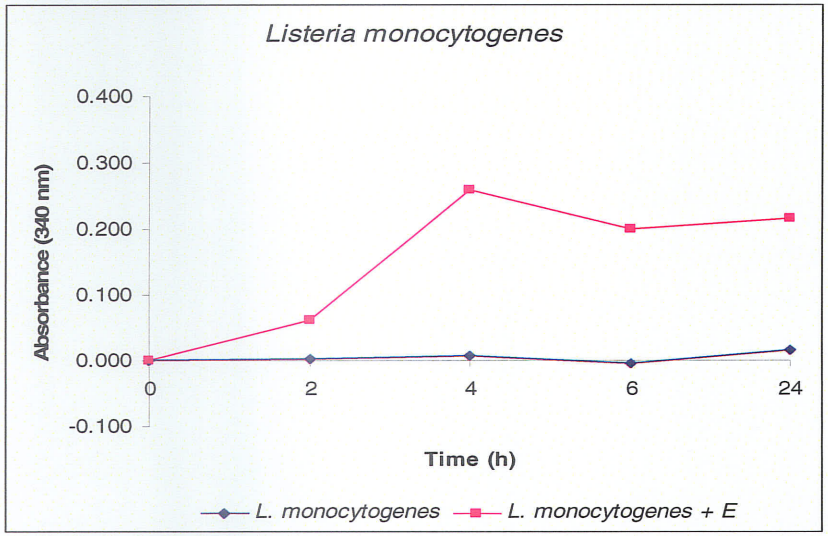
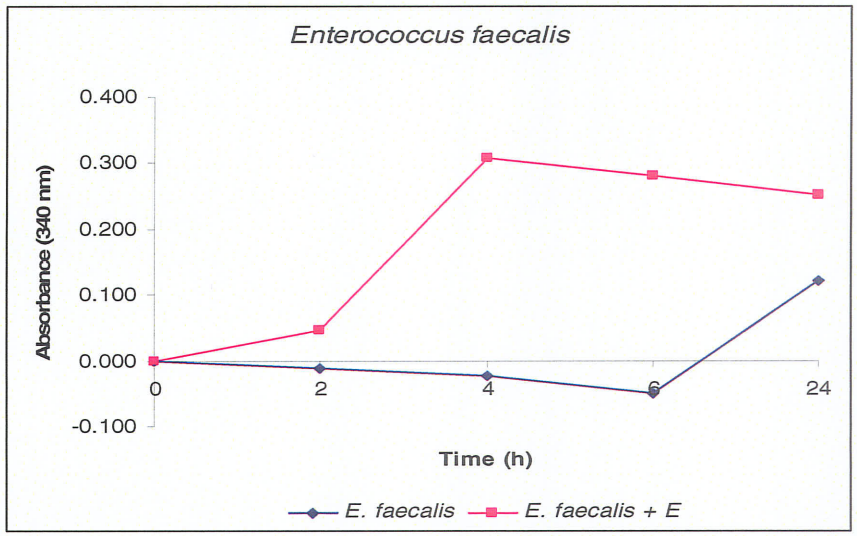
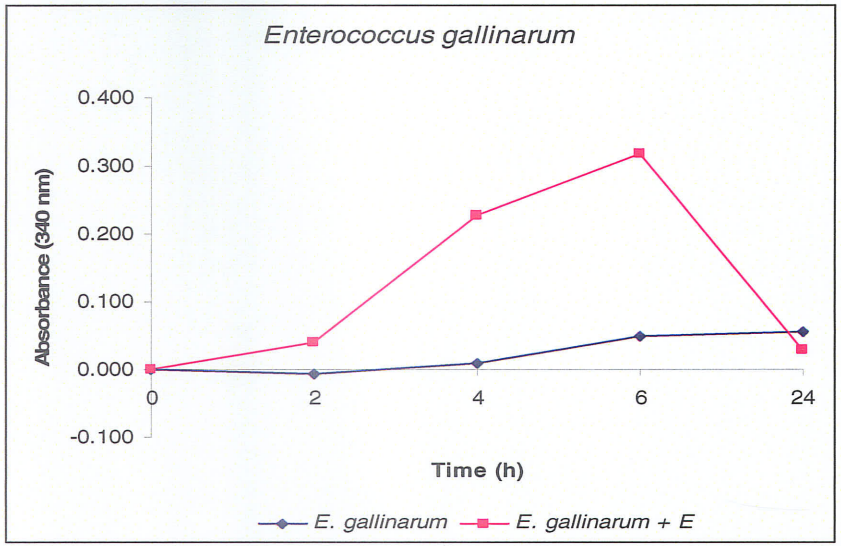
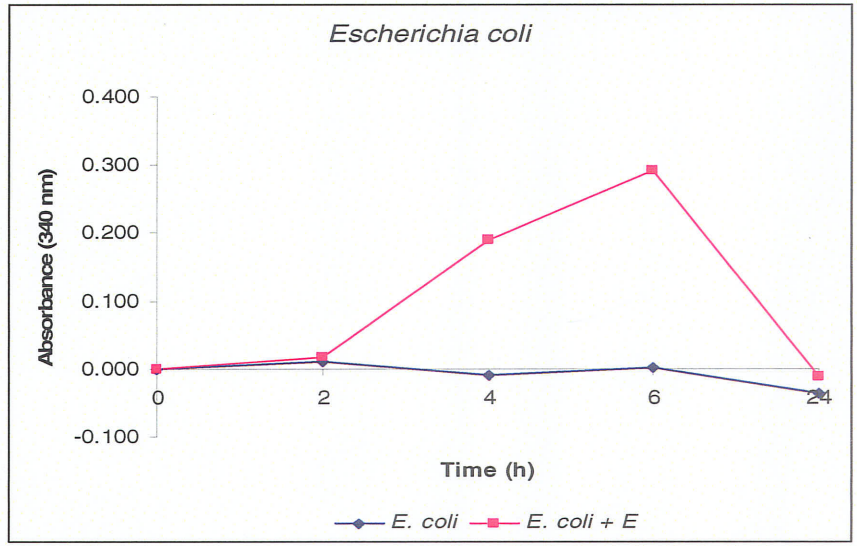


Figure 5. Bacterial growth in basal medium containing galactosidase enzyme (Copan α–gal) and non-enzyme treated raffinose.









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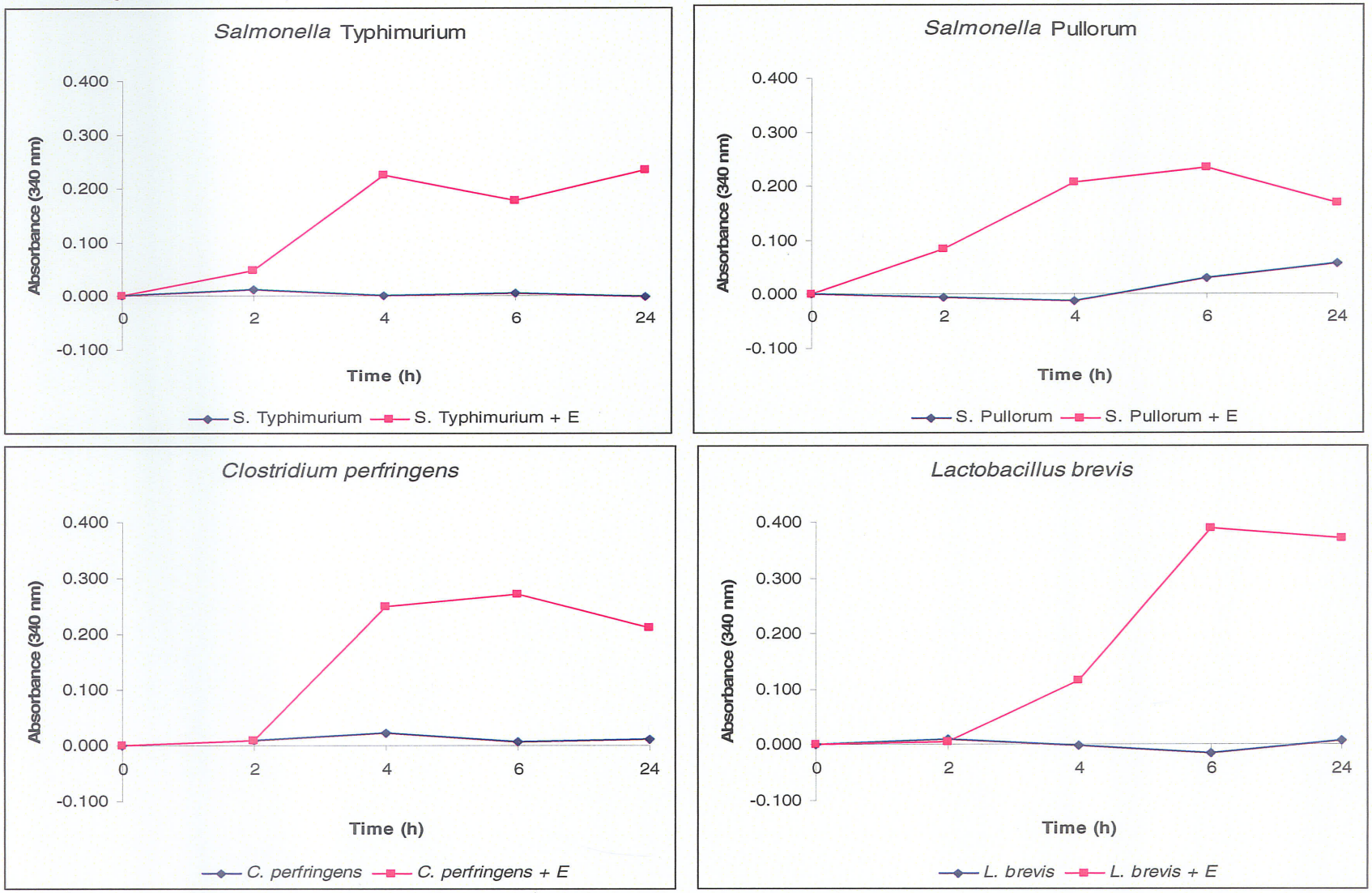

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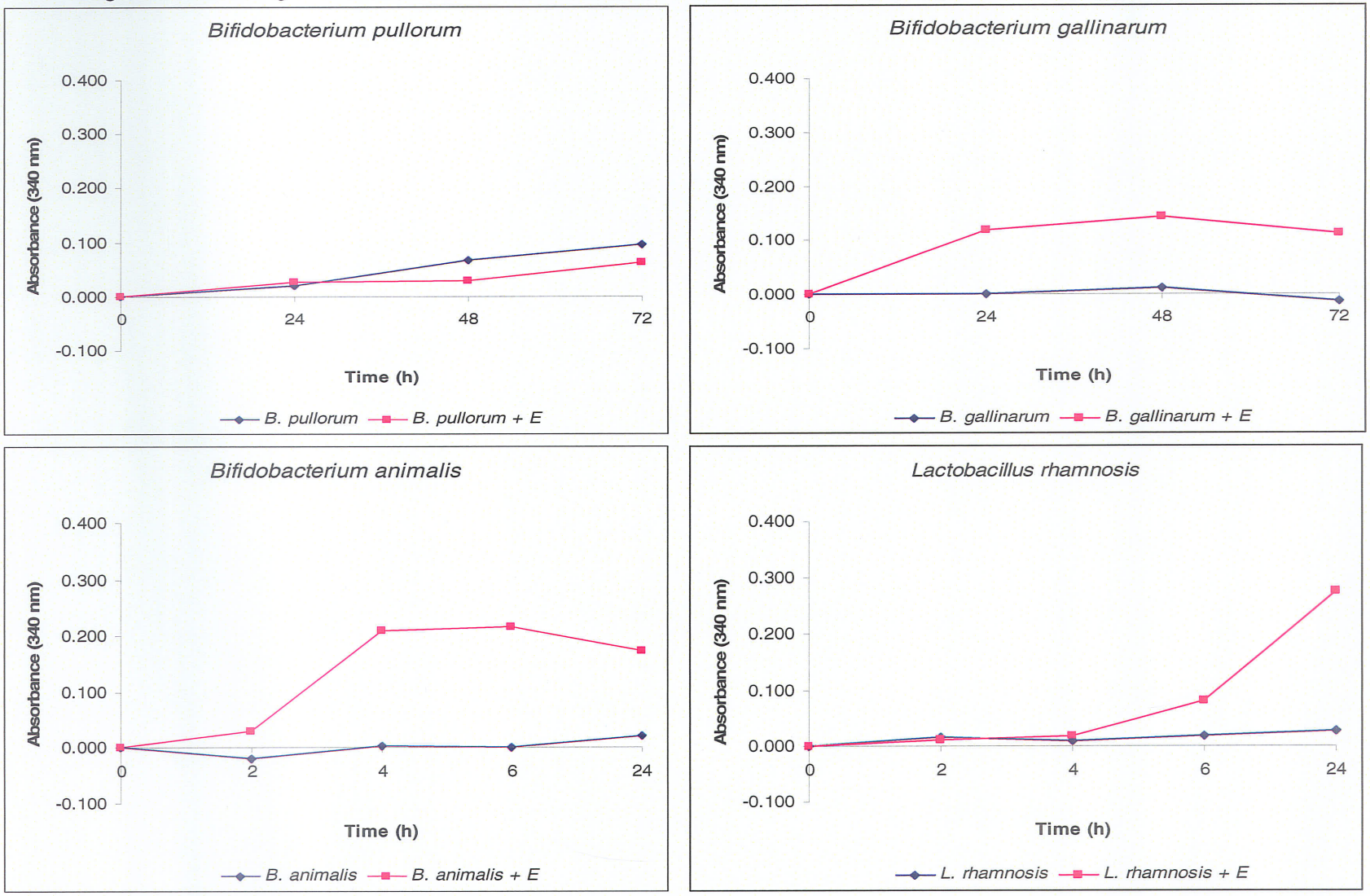

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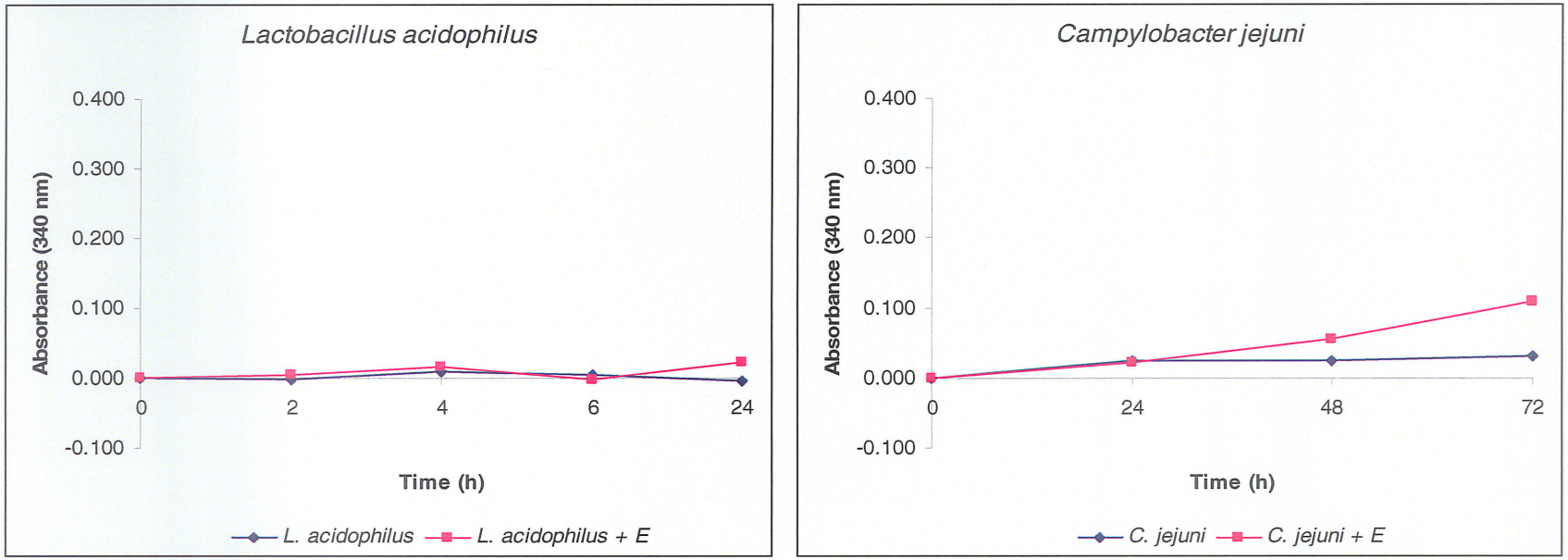
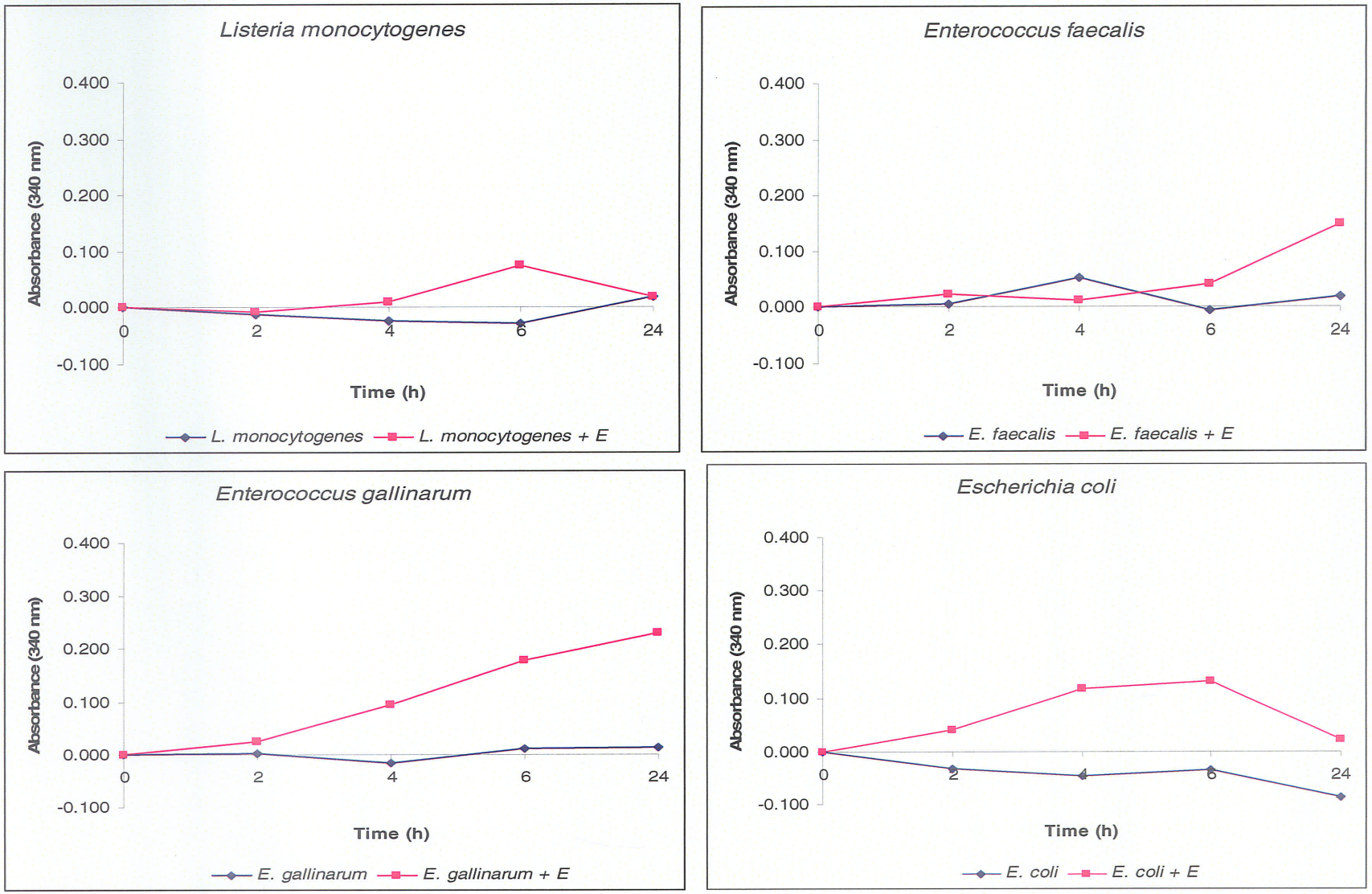


Figure 6. Bacterial growth in basal medium containing galactanase enzyme (Biogalactosidase 1000P) and non-enzyme treated guar gum.



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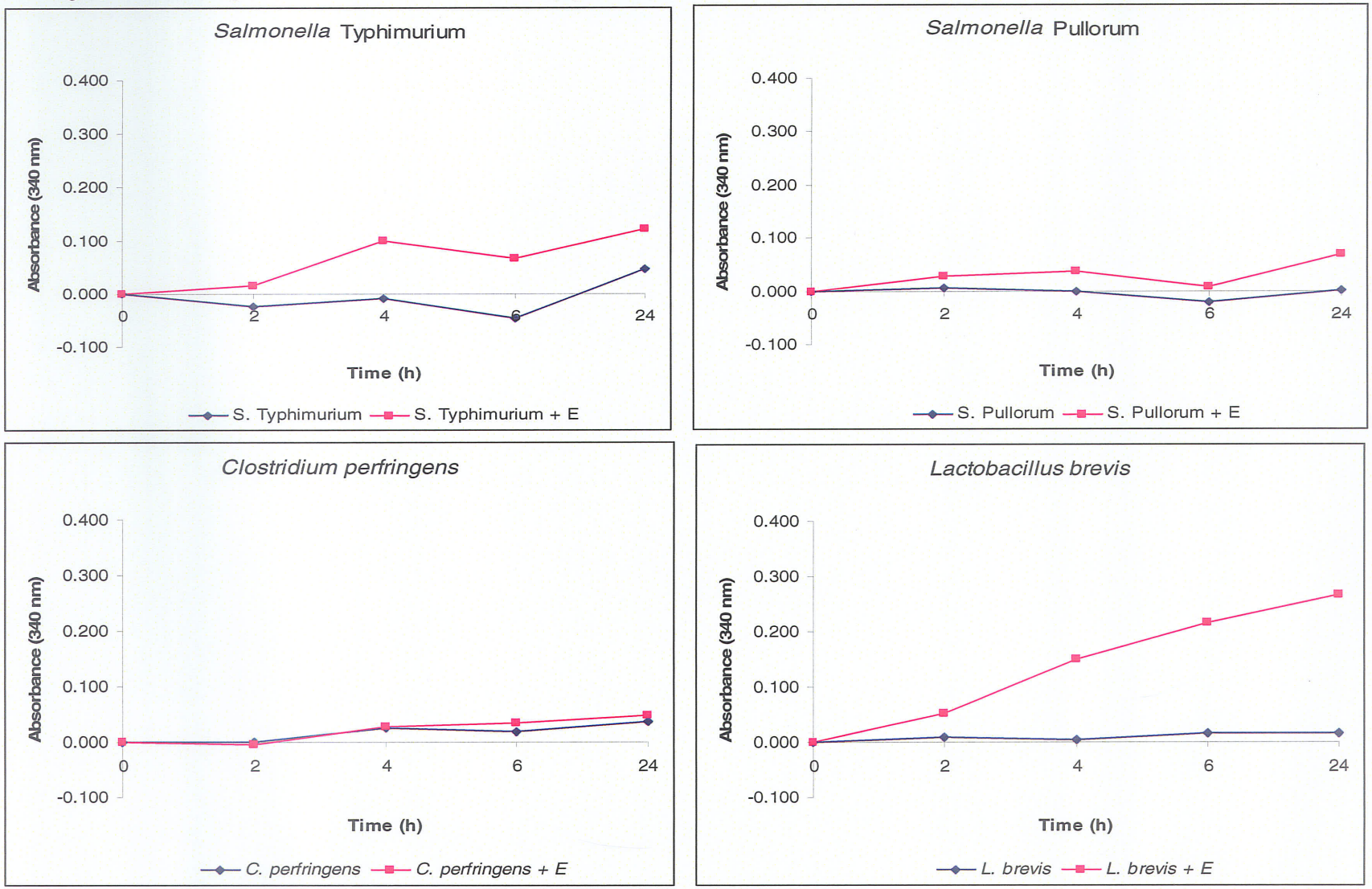
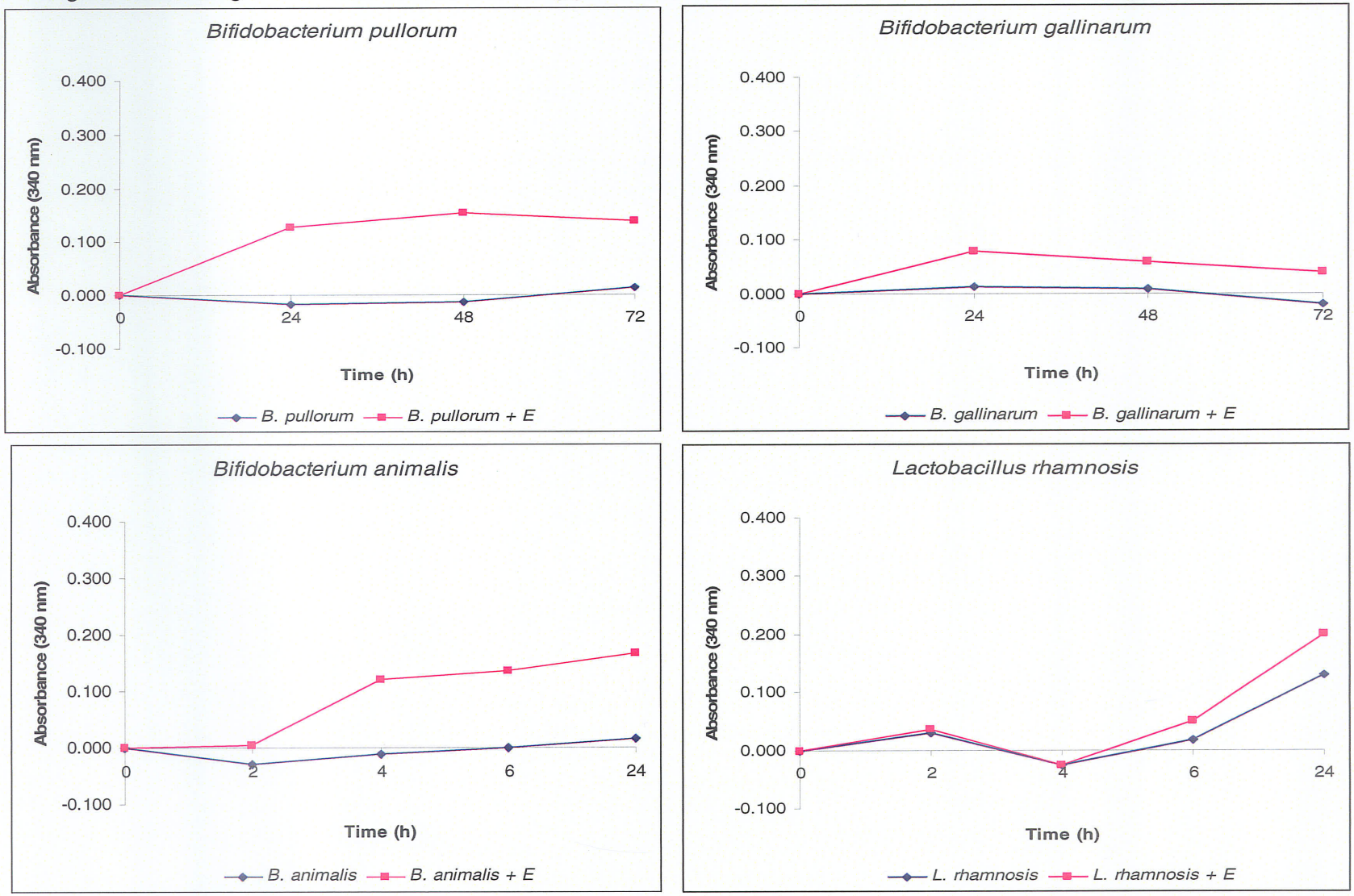


Figure 6. Bacterial growth in basal medium containing galactanase enzyme (Biogalactosidase 1000P) and non-enzyme treated guar gum.



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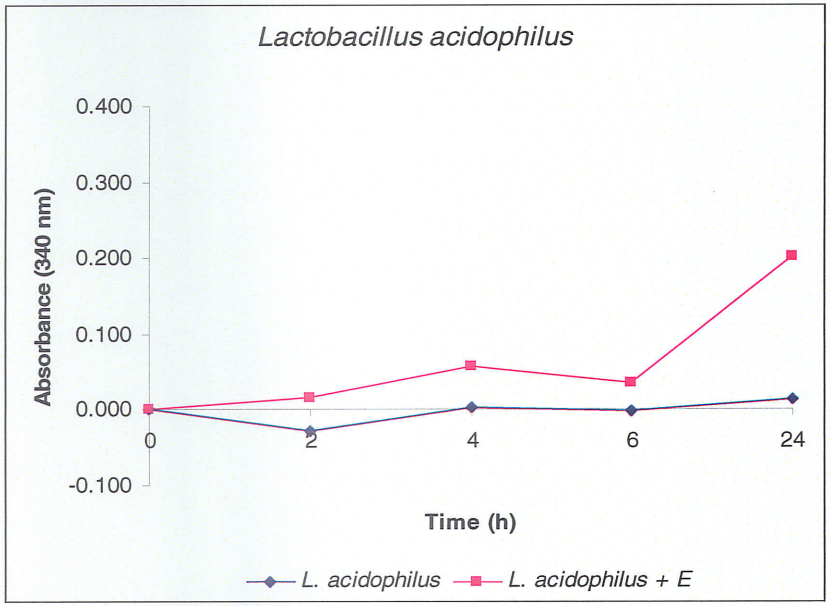

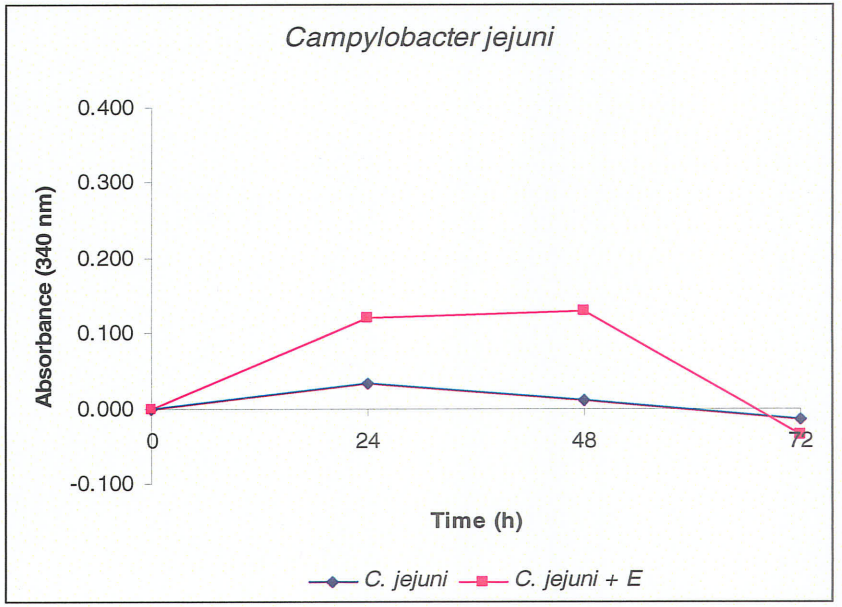

particular exhibited robust growth (≥ 1.5 log increases within 6 h). In the xylan-based medium *E. gallinarum*, *E. coli*, *Cl. perfringens* and *B. gallinarum* exhibited an increase in growth (≥ 1 log) as a result of enzyme supplementation (Table 10) while raffinose hydrolysis by galactosidase resulted in increased growth for most bacteria (Table 11). *L. monocytogenes*, *E. faecalis*, *Cl. perfringens*, *L. brevis* , *L. acidophilus* and *C. jejuni* in particular benefited from the presence of enzyme, exhibiting increases in growth of at least one log. Compared to α-galactosidase, inclusion of galactanase in a guar gum-based medium resulted in a reduced benefit (< 1 log increase) to most bacteria with the exception of *Cl. perfringens* and possibly *E. gallinarum* (Table 12).

Table 8. Changes in bacterial numbers during incubation[a] in β-glucan with or without glucanase A treatment.

Microorganism	Time (h)	Growth in β-glucan (Log_{10} CFU/mL)[b]		Difference[c] (Log_{10} CFU/mL)
		glucanase A	no enzyme	
Listeria monocytogenes	0	7.614	7.486	0.128
	6	8.255	8.093	0.162
	24	9.121	10.447	-1.326
Enterococcus faecalis	0	6.146	6.894	-0.748
	6	7.728	7.483	0.245
	24	7.973	7.505	0.468
Enterococcus gallinarum	0	6.769	6.787	-0.018
	6	7.815	7.520	0.295
	24	8.544	7.612	0.932
Escherichia coli	0	6.740	6.918	-0.178
	6	8.290	7.780	0.510
	24	9.320	9.079	0.241
Salmonella Typhimurium	0	6.543	6.621	-0.078
	6	8.730	8.600	0.130
	24	9.274	8.810	0.464
Salmonella Pullorum	0	7.732	7.916	-0.184
	6	8.728	8.766	-0.038
	24	9.330	9.193	0.137
Clostridium perfringens	0	7.274	7.161	0.113
	6	7.455	7.189	0.266
	24	7.872	7.068	0.804

Table 8. Changes in bacterial numbers during incubation[a] in β-glucan with or without glucanase A treatment.

Microorganism	Time (h)	Growth in β-glucan (Log_{10} CFU/mL)[b]		Difference[c] (Log_{10} CFU/mL)
		glucanase A	no enzyme	
Lactobacillus brevis	0	6.977	6.999	-0.022
	6	7.465	7.122	0.343
	24	8.488	8.256	0.232
Bifidobacterium pullorum	0	7.327	7.363	-0.036
	6	7.373	7.283	0.090
	24	8.041	7.365	0.676
Bifidobacterium gallinarum	0	7.316	7.306	0.010
	24	7.595	7.233	0.362
	48	8.166	7.269	0.897
Bifidobacteria animalis	0	7.370	7.316	0.054
	6	7.499	7.320	0.179
	24	7.722	7.330	0.392
Lactobacillus rhamnosis	0	7.123	7.038	-0.085
	6	7.422	7.390	-0.032
	24	7.835	7.690	0.145
Lactobacillus acidophilus	0	7.409	7.390	0.019
	6	7.560	7.453	0.107
	24	7.857	7.777	0.080
Campylobacter jejuni	0	7.244	7.289	-0.045
	6	7.347	7.321	0.026
	24	7.338	7.298	0.040

[a] Incubation at 40°C ≤ 48 h.

[b] Plate count growth (log_{10} CFU/mL) average of duplicates.

[c] Difference in growth (growth with enzyme minus growth without enzyme).

Table 9. Changes in bacterial numbers during incubation[a] in β-glucan with or without glucanase B treatment.

Microorganism	Time (h)	Growth in β-glucan (Log_{10} CFU/mL)[b]		Difference[c] (Log_{10} CFU/mL)
		glucanase B	no enzyme	
Listeria monocytogenes	0	7.528	7.626	-0.098
	6	8.615	6.720	1.895
	24	9.618	8.714	0.904
Enterococcus faecalis	0	7.221	6.750	0.471
	6	8.568	8.267	0.301
	24	8.658	8.760	-0.102
Enterococcus gallinarum	0	6.241	6.170	0.071
	6	10.436	9.424	1.012
	24	8.714	8.525	0.189
Escherichia coli	0	7.588	7.607	-0.019
	6	9.050	8.458	0.592
	24	8.903	8.767	0.136
Salmonella Typhimurium	0	7.695	7.748	-0.053
	6	10.444	8.436	2.008
	24	10.458	10.137	0.321
Salmonella Pullorum	0	7.304	7.243	0.061
	6	9.678	7.899	1.779
	24	9.324	9.234	0.090
Clostridium perfringens	0	7.130	7.161	-0.031
	6	7.210	7.098	0.112
	24	7.269	7.068	0.201

Table 9. Changes in bacterial numbers during incubation[a] in β-glucan with or without glucanase B treatment.

Microorganism	Time (h)	Growth in β-glucan (Log_{10} CFU/mL)[b]		Difference[c] (Log_{10} CFU/mL)
		glucanase B	no enzyme	
Lactobacillus brevis	0	7.427	7.470	-0.043
	6	8.041	7.479	0.562
	24	8.600	7.498	1.102
Bifidobacterium pullorum	24	6.477	5.653	0.824
	48	5.653	5.653	0.000
	72	6.891	5.651	1.240
Bifidobacterium gallinarum	0	6.616	6.927	-0.311
	24	8.489	8.146	0.343
	48	8.743	8.253	0.490
Bifidobacteria animalis	0	7.318	7.083	0.235
	6	8.217	7.648	0.569
	24	8.276	7.735	0.541
Lactobacillus rhamnosis	0	7.149	7.199	-0.050
	6	7.702	7.021	0.681
	24	7.766	6.911	0.855
Lactobacillus acidophilus	0	6.017	6.996	-0.979
	6	7.441	7.352	0.089
	24	6.782	6.594	0.188
Campylobacter jejuni	0	7.077	7.022	0.055
	6	7.698	7.174	0.524
	24	7.844	7.213	0.631

[a] Incubation at 40°C ≤ 48 h.

[b] Plate count growth (log_{10} CFU/mL) average of duplicates.

[c] Difference in growth (growth with enzyme minus growth without enzyme).

Table 10. Changes in bacterial numbers during incubation[a] in xylan with or without xylanase treatment.

Microorganism	Time (h)	Growth in xylan (Log_{10} CFU/mL)[b]		Difference[c] (Log_{10} CFU/mL)
		xylanase	no enzyme	
Listeria monocytogenes	0	7.009	6.960	0.049
	6	9.549	9.640	-0.091
	24	9.760	9.789	-0.029
Enterococcus faecalis	0	6.491	6.915	-0.424
	6	9.117	9.431	-0.314
	24	9.900	9.581	0.319
Enterococcus gallinarum	0	6.799	6.613	0.186
	6	8.332	7.000	1.332
	24	7.695	7.563	0.132
Escherichia coli	0	7.732	7.305	0.427
	6	8.342	8.093	0.249
	24	9.848	8.185	1.663
Salmonella Typhimurium	0	6.716	7.072	-0.356
	6	9.618	9.771	-0.153
	24	9.033	8.672	0.361
Salmonella Pullorum	0	7.679	7.704	-0.025
	6	8.577	8.568	0.009
	24	9.848	9.526	0.322
Clostridium perfringens	0	6.806	6.697	0.109
	6	7.332	6.688	0.644
	24	9.303	7.605	1.698

Table 10. Changes in bacterial numbers during incubation[a] in xylan with or without xylanase treatment.

Microorganism	Time (h)	Growth in xylan (Log_{10} CFU/mL)[b]		Difference[c] (Log_{10} CFU/mL)
		xylanase	no enzyme	
Lactobacillus brevis	0	7.270	7.685	-0.415
	6	7.862	7.724	0.138
	24	7.628	7.512	0.116
Bifidobacterium pullorum	24	6.775	6.823	-0.048
	48	7.403	7.398	0.005
	72	6.114	6.130	-0.016
Bifidobacterium gallinarum	0	6.566	6.869	-0.303
	24	8.236	7.948	0.288
	48	10.435	8.382	2.053
Bifidobacteria animalis	0	5.903	5.950	-0.047
	6	8.170	7.699	0.471
	24	7.878	7.739	0.139
Lactobacillus rhamnosis	0	5.767	5.796	-0.029
	6	7.037	6.516	0.521
	24	7.182	7.124	0.058
Lactobacillus acidophilus	0	7.146	7.196	-0.050
	6	6.334	6.290	0.044
	24	5.616	6.086	-0.470
Campylobacter jejuni	0	6.163	6.257	-0.094
	6	7.023	6.906	0.117
	24	7.966	7.122	0.844

[a] Incubation at 40°C ≤ 48 h.

[b] Plate count growth (log_{10} CFU/mL) average of duplicates.

[c] Difference in growth (growth with enzyme minus growth without enzyme).

Table 11. Changes in bacterial numbers during incubation[a] in raffinose with or without α-galactosidase treatment.

Microorganism	Time (h)	Growth in raffinose (Log_{10} CFU/mL)[b]		Difference[c] (Log_{10} CFU/mL)
		α-galactosidase	no enzyme	
Listeria monocytogenes	0	7.049	7.444	-0.395
	6	9.851	8.633	1.218
	24	10.312	9.004	1.308
Enterococcus faecalis	0	6.848	6.723	0.125
	6	8.431	7.754	0.677
	24	11.334	10.170	1.164
Enterococcus gallinarum	0	6.658	6.775	-0.117
	6	9.330	8.494	0.836
	24	9.415	8.917	0.498
Escherichia coli	0	7.279	7.158	0.121
	6	8.601	8.114	0.487
	24	9.240	9.000	0.240
Salmonella Typhimurium	0	7.064	7.146	-0.082
	6	9.025	8.438	0.587
	24	9.413	9.196	0.217
Salmonella Pullorum	0	7.246	7.173	0.073
	6	8.678	8.212	0.466
	24	9.312	9.267	0.045
Clostridium perfringens	0	6.531	6.477	0.054
	6	8.111	7.079	1.032
	24	7.641	7.348	0.293

Table 11. Changes in bacterial numbers during incubation[a] in raffinose with or without α-galactosidase treatment.

Microorganism	Time (h)	Growth in raffinose (Log_{10} CFU/mL)[b]		Difference[c] (Log_{10} CFU/mL)
		α-galactosidase	no enzyme	
Lactobacillus brevis	0	6.789	6.658	0.131
	6	8.504	7.480	1.024
	24	8.633	7.714	0.919
Bifidobacterium pullorum	24	5.653	6.061	-0.408
	48	5.544	5.778	-0.234
	72	8.607	8.097	0.510
Bifidobacterium gallinarum	0	6.505	6.371	0.134
	24	8.643	8.582	0.061
	48	8.430	8.258	0.172
Bifidobacteria animalis	0	7.228	7.185	0.043
	6	8.045	7.508	0.537
	24	8.149	7.594	0.555
Lactobacillus rhamnosis	0	7.143	7.041	0.102
	6	7.057	6.984	0.073
	24	7.594	6.847	0.747
Lactobacillus acidophilus	0	7.029	6.857	0.172
	6	8.130	7.117	1.013
	24	6.823	6.265	0.558
Campylobacter jejuni	0	7.044	7.011	0.033
	6	7.908	7.223	0.685
	24	8.122	7.042	1.080

[a] Incubation at 40°C ≤ 48 h.

[b] Plate count growth (log_{10} CFU/mL) average of duplicates.

[c] Difference in growth (growth with enzyme minus growth without enzyme).

Table 12. Changes in bacterial numbers during incubation[a] in guar gum with or without galactanase treatment.

Microorganism	Time (h)	Growth in guar gum (Log_{10} CFU/mL)[b]		Difference[c] (Log_{10} CFU/mL)
		galactanase	no enzyme	
Listeria monocytogenes	0	7.614	7.486	0.128
	6	8.255	8.093	0.162
	24	9.121	10.447	-1.326
Enterococcus faecalis	0	6.146	6.894	-0.748
	6	7.728	7.483	0.245
	24	7.973	7.505	0.468
Enterococcus gallinarum	0	6.769	6.787	-0.018
	6	7.815	7.520	0.295
	24	8.544	7.612	0.932
Escherichia coli	0	6.740	6.918	-0.178
	6	8.290	7.780	0.510
	24	9.320	9.079	0.241
Salmonella Typhimurium	0	6.543	6.621	-0.078
	6	8.730	8.600	0.130
	24	9.274	8.810	0.464
Salmonella Pullorum	0	7.732	7.916	-0.184
	6	8.728	8.766	-0.038
	24	9.330	9.193	0.137
Clostridium perfringens	0	6.886	6.538	0.348
	6	7.493	6.398	1.095
	24	8.223	7.600	0.623

Table 12. Changes in bacterial numbers during incubation[a] in guar gum with or without galactanase treatment.

Microorganism	Time (h)	Growth in guar gum (Log_{10} CFU/mL)[b]		Difference[c] (Log_{10} CFU/mL)
		galactanase	no enzyme	
Lactobacillus brevis	0	7.225	7.415	-0.190
	6	8.228	7.699	0.529
	24	7.903	7.744	0.159
Bifidobacterium pullorum	24	7.111	6.481	0.630
	48	8.130	7.583	0.547
	72	7.702	8.204	-0.502
Bifidobacterium gallinarum	0	6.963	6.600	0.363
	24	8.826	8.072	0.754
	48	9.324	9.455	-0.131
Bifidobacteria animalis	0	6.577	7.045	-0.468
	6	8.033	7.560	0.473
	24	8.025	7.905	0.120
Lactobacillus rhamnosis	0	7.220	7.121	0.099
	6	7.367	7.991	-0.624
	24	7.556	7.025	0.531
Lactobacillus acidophilus	0	6.921	6.903	0.018
	6	6.885	6.949	-0.064
	24	8.396	7.550	0.846
Campylobacter jejuni	0	7.164	7.023	0.141
	6	7.453	7.088	0.365
	24	7.877	7.453	0.424

[a] Incubation at 40°C ≤ 48 h.

[b] Plate count growth (log_{10} CFU/mL) average of duplicates.

[c] Difference in growth (growth with enzyme minus growth without enzyme).

3.4.4 Bacterial growth in ethanol-extracted soybean and canola meals with or without enzyme supplementation

Bacterial growth in ethanol-extracted soybean and canola meals with or without exogenous enzyme supplementation is shown in Table 13. The majority of microorganisms, especially when grown on soybean meal for 6 h, showed little or no growth enhancement after enzyme supplementation even after 24 h of incubation.

Microorganisms which did exhibit a significant increase in growth after 6 h of incubation included *Salmonella* Typhimurium with soybean meal, *Campylobacter jejuni* with both soybean and canola meals, and *Lactobacillus brevis* and *Bifidobacterium pullorum* with canola meal. *Escherichia coli* also showed a slight, but significant increase in growth on canola meal after 24 h. At 24 h only *Salmonella* Typhimurium continued to exhibit increased growth on soybean meal.

3.4.5 Broiler chicken experiment

The recovery of *E. coli* from the caecal and ileal contents of broilers maintained on various diets is shown in Figure 7. Birds fed diets containing either wheat or corn contained significantly lower ($p < 0.05$) levels of *E. coli* in their caecal contents as a result of enzyme and/or probiotic supplementation. In

Table 13. Microbial growth on ethanol extracted soybean and canola meals with or without enzyme supplementation at 40°C.

Microorganism	Population (log_{10} CFU/mL) at time											
	6 h						24 h					
	SBM[1]	SBME[2]	D[3]	CM[4]	CME[5]	D[6]	SBM	SBME	D	CM	CME	D
Enterococcus faecalis	0.278	0.205	-0.073[a]	0.159	0.137	-0.022[a]	0.215	0.268	0.053[a]	0.268	0.121	-0.147[a]
Escherichia coli	0.067	0.113	0.046[a]	-0.045	0.115	0.160[a]	0.074	0.138	0.064[a]	-0.051	0.130	0.181[b]
Salmonella Typhimurium	0.211	0.412	0.201[b]	0.312	0.298	-0.014[a]	0.261	0.452	0.191[b]	0.359	0.346	-0.013[a]
Clostridium perfringens	0.168	0.133	-0.035[a]	0.132	0.203	-0.071[a]	0.177	0.152	-0.025[a]	0.147	0.241	0.094[a]
Campylobacter jejuni	0.144	0.446	0.302[b]	0.120	0.430	0.310[b]	0.569	0.717	0.148[a]	0.398	0.411	0.013[a]
Lactobacillus brevis	0.298	0.244	-0.054[a]	0.087	0.665	0.578[c]	0.349	0.201	-0.148[a]	0.170	0.224	0.054[a]
Bifidobacterium pullorum	0.143	0.132	-0.011[a]	0.455	0.988	0.533[c]	0.384	0.043	-0.341[b]	1.370	1.404	0.034[a]

[1] SBM = growth on soybean meal minus growth on basal medium; values are averages of triplicates each in duplicate (n=6)
[2] SBME = growth on soybean meal with enzyme supplementation minus growth on basal medium; averages of triplicates each in duplicate (n=6)
[3] D = difference in growth SBME-SBM (log_{10} CFU/mL)
[4] CM = growth on canola meal minus growth on basal medium; values are averages of triplicates each in duplicate (n=6)
[5] CME = growth on canola meal with enzyme supplementation minus growth on basal medium; averages of triplicates each in duplicate (n=6)
[6] D = difference in growth CME-CM (log_{10} CFU/mL)
[a-c] Values in columns with different letter superscripts are significantly different ($P<0.05$)

contrast, inclusion of glucanase in the barley-based diets resulted in significantly higher levels of *E. coli* in the caeca. A reduction in *E. coli* was also observed in the ileum but only in the corn-based diet containing the probiotic. Overall, *E. coli* levels were at least 1 log higher in the caeca compared to the ileum for all diets and protocols. Coliform results were not recorded since nearly all colonies growing on the Petrifim® were *E. coli* (blue colored colonies surrounded by gas).

Enterococci levels (Figure 8) in the ileum were not significantly different ($p > 0.05$) regardless of diet. In the caeca, only the barley based diets treated with enzyme and/or probiotic yielded significantly ($p < 0.005$) higher levels of enterococci.

Aerobic spore levels were significantly higher ($p < 0.05$) in the caeca of all poultry fed diets supplemented with enzyme (Figure 9). In addition, poultry fed the corn-based diet containing both enzyme and probiotic exhibited significantly ($p < 0.05$) higher spore levels compared to poultry fed corn supplemented only with enzyme. In contrast, a significant increase in aerobic spores was only observed in the ileum of poultry which were maintained on barley and corn based diets. Inclusion of probiotics into feeds did not result in higher spore levels in the ileum when compared to feeds containing only multicarbohydrase enzyme.

Addition of enzyme to all feeds (Figure 10) resulted in significantly ($p < 0.05$) higher levels of anaerobic spores in the caeca. In the case of the corn diet, addition of the probiotic resulted in a further increase in the level of

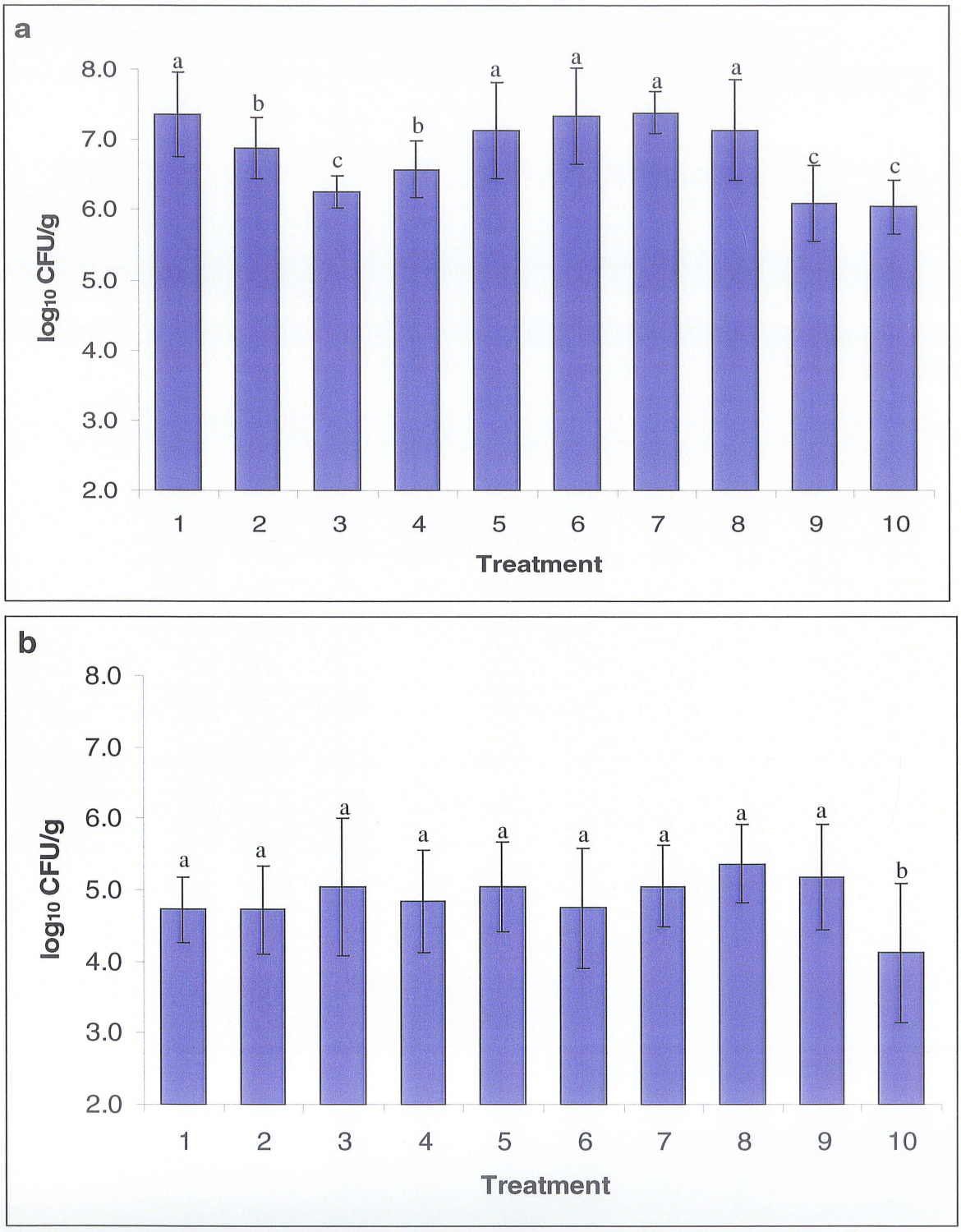

Figure 7. Recovery of *Escherichia coli* from (a) caeca and (b) ileum contents of chickens from different treatment groups. Values represent the means of 9 samples each performed in duplicate (n=18) ± standard deviation. Treatments with different letters are significantly different ($P<0.05$).
Treatments: 1 – wheat-based; 2 – wheat-based + xylanase; 3 – wheat-based + xylanase + probiotic; 4 – barley-based; 5 – barley-based + glucanase A; 6 – barley-based + glucanase B; 7 – barley-based + glucanase B + probiotic; 8 – corn-based; 9 – corn-based + multicarbohydrase; 10 – corn-based + multicarbohydrase + probiotic.

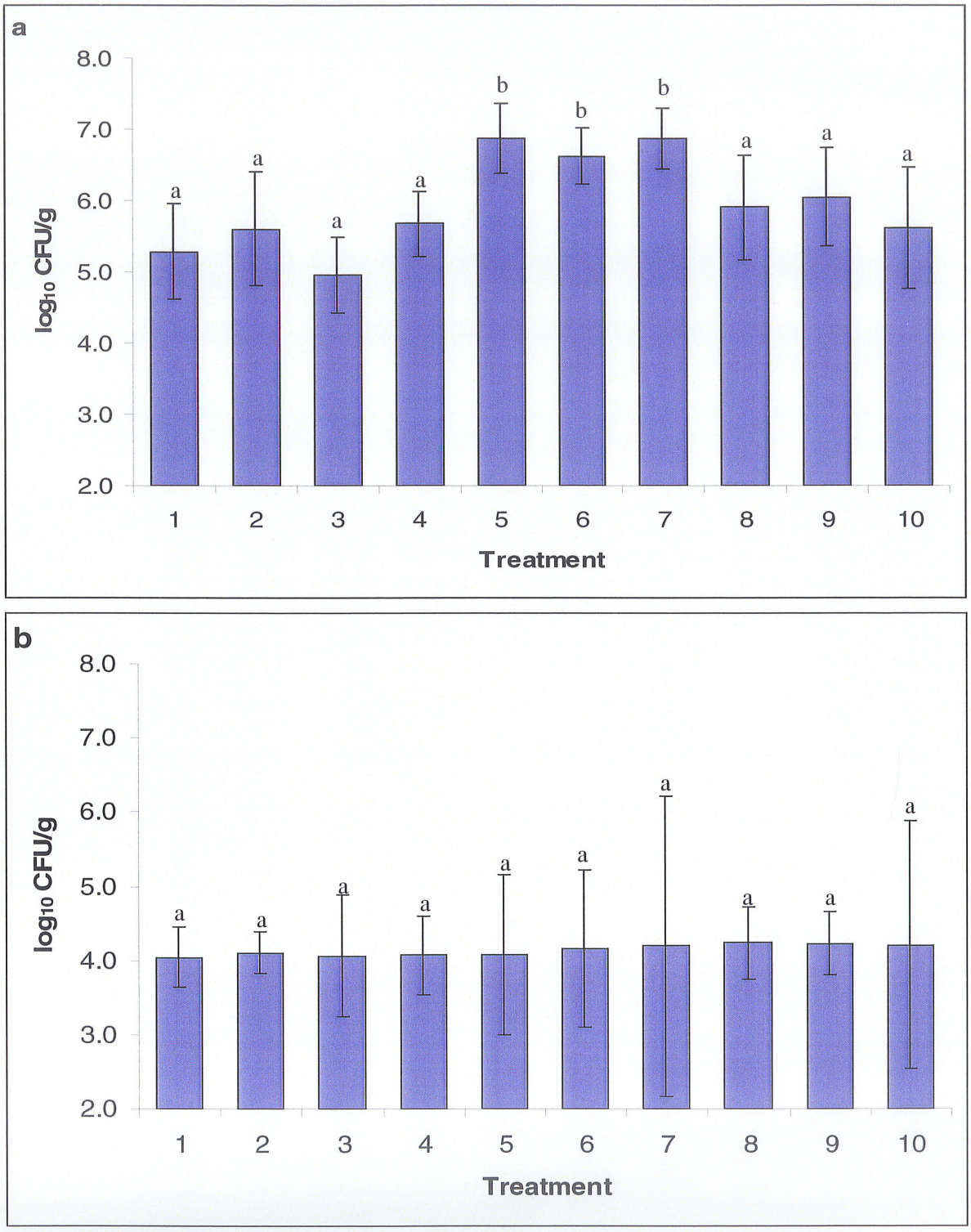

Figure 8. Recovery of enterococci from (a) caeca and (b) ileum contents of chickens from different treatment groups. Values represent the means of 9 samples each performed in triplicate (n=27) ± standard deviation. Treatments with different letters are significantly different ($P<0.05$).
Treatments: 1 – wheat-based; 2 – wheat-based + xylanase; 3 – wheat-based + xylanase + probiotic; 4 – barley-based; 5 – barley-based + glucanase A; 6 – barley-based + glucanase B; 7 – barley-based + glucanase B + probiotic; 8 – corn-based; 9 – corn-based + multicarbohydrase; 10 – corn-based + multicarbohydrase + probiotic.

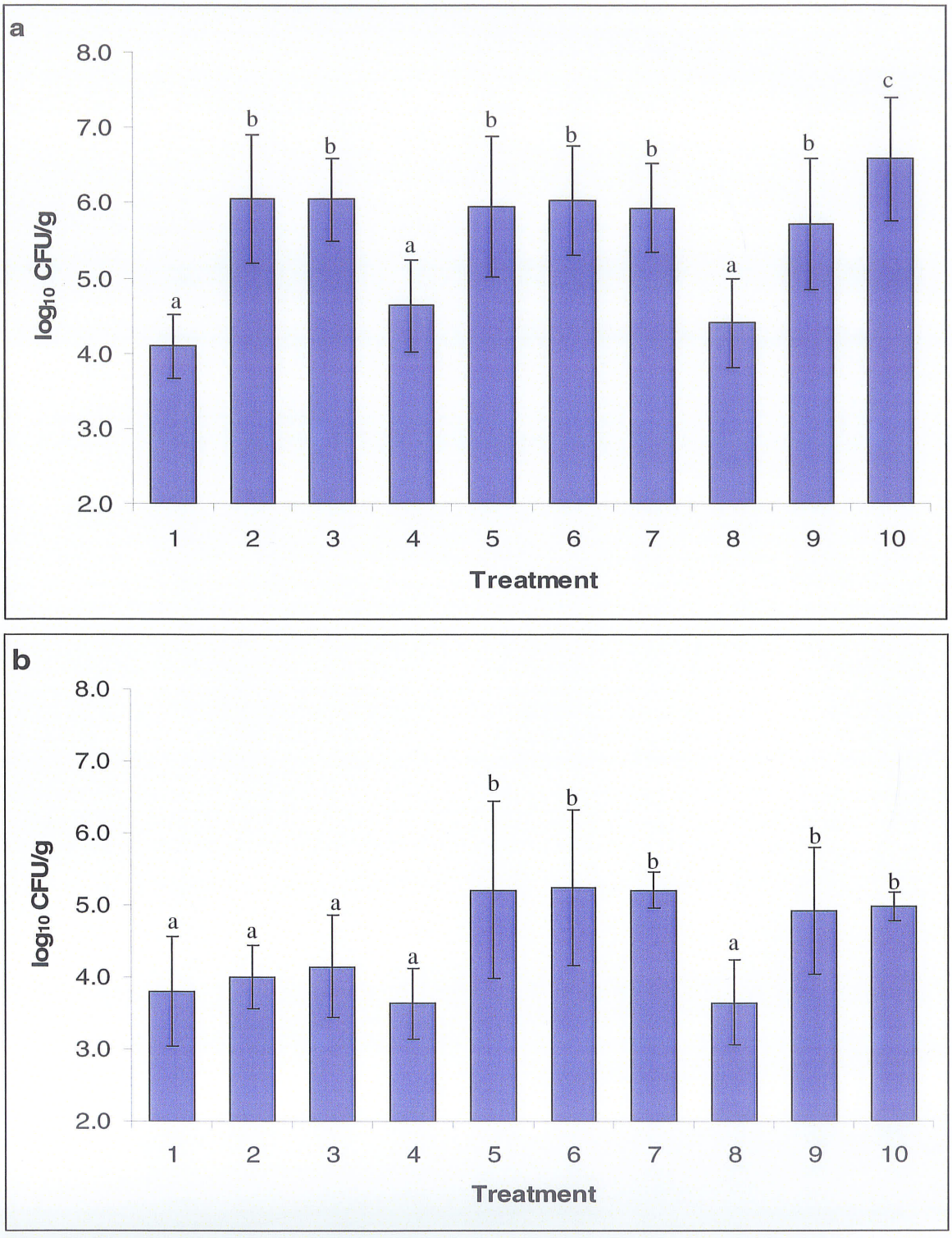

Figure 9. Recovery of aerobic spore-formers from (a) caeca and (b) ileum contents of chickens from different treatment groups. Values represent the means of 9 samples each performed in triplicate (n=27) ± standard deviation. Treatments with different letters are significantly different ($P<0.05$).
Treatments: 1 – wheat-based; 2 – wheat-based + xylanase; 3 – wheat-based + xylanase + probiotic; 4 – barley-based; 5 – barley-based + glucanase A; 6 – barley-based + glucanase B; 7 – barley-based + glucanase B + probiotic; 8 – corn-based; 9 – corn-based + multicarbohydrase; 10 – corn-based + multicarbohydrase + probiotic.

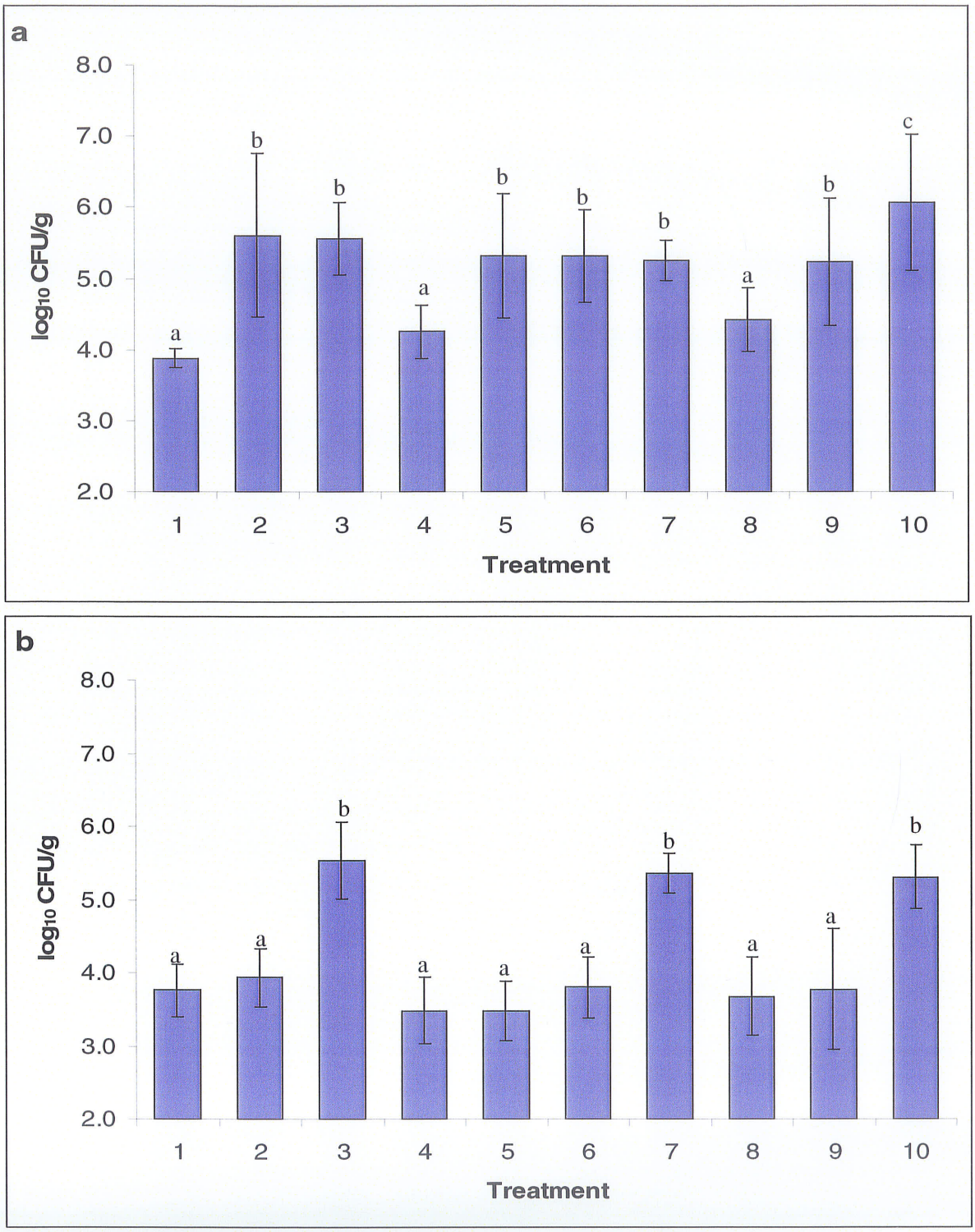

Figure 10. Recovery of anaerobic spore-formers from (a) caeca and (b) ileum contents of chickens from different treatment groups. Values represent the means of 9 samples each performed in triplicate (n=27) ± standard deviation. Treatments with different letters are significantly different ($P<0.05$).
Treatments: 1 – wheat-based; 2 – wheat-based + xylanase; 3 – wheat-based + xylanase + probiotic; 4 – barley-based; 5 – barley-based + glucanase A; 6 – barley-based + glucanase B; 7 – barley-based + glucanase B + probiotic; 8 – corn-based; 9 – corn-based + multicarbohydrase; 10 – corn-based + multicarbohydrase + probiotic.

anaerobic spores. In the ileum significant ($p < 0.05$) increases in anaerobic spore levels occurred only in diets supplemented with the probiotic. In both the caeca and ileum no significant ($p > 0.05$) differences in *E. coli*, enterococci and spores levels were observed between any of the treatments containing either glucanase A or glucanase B.

Feed intake, body weight gains and feed to gain ratios of chickens fed various diets are presented in Table 14. Body weight gain significantly ($p < 0.05$) increased with addition of the exogenous enzyme in all three diets. However, no further increases were observed upon addition of the probiotic. The feed to gain ratios decreased with enzyme addition for the wheat and corn-based diets and, similar to the body weight gain, did not decrease further with addition of the probiotic. The feed to gain ratio for the barley based diet decreased somewhat with glucanase A, but not with addition of glucanase B. Contrary to the other two diets, the feed to gain ratios for the barley diet decreased when supplemented with the probiotic.

Table 14. Effect of enzyme and probiotic additions on growth performance of broiler chickens (5-18d) fed different diets.

Diet	Treatment	Feed intake (g/bird)	Body weight gain g/bird/12d	Feed/gain ratio
Wheat/soybean/canola meal	1. Control (no additive)	530.8	374.7[b]	1.416[a]
	2. Xylanase	527.7	397.0[a]	1.329[b]
	3. Xylanase + Probiotic[1]	518.2	390.4[a,b]	1.328[b]
SEM[2]		10.2	6.5	0.013
Barley/soybean/canola meal	4. Control (no additive)	529.4	412.5[b]	1.284[a]
	5. Glucanase A	552.5	440.9[a]	1.254[a,b]
	6. Glucanase B	563.2	437.4[a]	1.288[a]
	7. Glucanase B + Probiotic[1]	530.1	432.2[a,b]	1.228[b]
SEM		11.4	9.2	0.014
Corn/soybean/canola meal	8. Control (no additive)	548.0	427.6[b]	1.284[a]
	9. Multi-carbohydrase enzyme[3]	547.7	440.8[a]	1.241[b]
	10. Multi-carbohydrase enzyme + Probiotic[1]	550.1	437.8[a]	1.257[a,b]
SEM		6.7	7.4	0.012

[1] Combination of heat-stable lactic acid producing *Bacillus subtilis* and *Bacillus licheniformis*.
[2] SEM = standard error of the mean
[3] Cocktail of cell wall degrading activities (1000 U xylanase, 400 U glucanase, 1000 U pectinase, 120 U cellulase, 280 U mannanase and 180 U galactanase)
[a-b] Values in columns with different letter superscripts are significantly different ($P<0.05$)

3.5 DISCUSSION

3.5.1 Bacterial utilization of enzyme or non-enzyme treated oligosaccharides and NSP substrates

Preliminary studies evaluated the growth of pure cultures in a basal medium containing specified monosaccharides. The monosaccharides represent some of the component sugars of the feed ingredients used in this study which could be released following exogenous enzyme addition. Based on absorbance studies all target microorganisms exhibited varying degrees of growth in the monosaccharides provided, however, none appeared to utilize arabinose. Based on the in *vitro* assay, arabinose moieties contained in feed ingredients and liberated by polysaccharide depolymerization, would not be expected to be utilized to any great extent by any of the target bacteria, at least within a 5-6 hour period. This time frame represents a reasonable mean retention time for digesta in the poultry gut (Danicke et al. 1999), considering that Coon et al. (1990) reported transit times of approximately one hour with chickens fed a soybean-meal diet. However, how accurately these results reflect *in vivo* conditions is unknown. The intestinal tract of domestic animals plays an important role in digestion and complex bacterial interactions including symbiosis and antagonism are difficult to reproduce *in vitro*. Also, it is well recognized that many other, perhaps unidentified species of bacteria associated with the GIT would be capable of utilizing arabinose.

The inclusion of commercial enzymes with feed ingredients or substrates resulted in some interesting microbial growth profiles. In particular, the use of two glucanases, one that reduced viscosity but produced little free glucose and the other that produced large amounts of glucose, resulted in obvious differences in growth levels among some bacteria. Ostensibly the higher levels of free simple sugars released by the latter glucanase were responsible for the increases in growth. Unfortunately increases in growth were observed by both probiotic-like bacteria (*Lactobacillus* and *Bifidobacteria*) and pathogens (*L. monocytogenes*, *Salmonella* and *Campylobacter*). Overall, enzymes that produced the highest level of free glucose: glucanase B (859 mg/g) and α–galactosidase (479 mg/g) appeared to result in the largest number of bacterial genera yielding a growth increase ≥ 0.5 log within the specified time periods. This was expected since simple sugars are the most easily fermented by microorganisms in the GIT. Overall, none of the enzyme-treated substrates provided a clear advantage with respect to enhanced growth of LAB or decreased growth of pathogens.

Profiling microorganisms based on their assimilation of simple sugars using optical density did not always correlate with growth (plate counts or optical density) on enzyme-supplemented substrates. For example, based on the component sugar profile using xylanase, approximately 97 mg/g xylose was produced. Since *E. gallinarum* and *S.* Pullorum both exhibited growth on xylose, it was expected that both bacteria would also grow on enzyme-supplemented xylan. Although *E. gallinarum* exhibited enhanced growth due to the enzyme addition *S.* Pullorum did not. In addition, *Cl. perfringens* which yielded no growth

on xylose, exhibited enhanced growth on enzyme-supplemented xylan, possibly due to the low amounts of glucose also released by xylanase.

It is possible that discrepancies occurred because some cultures were capable of growing equally well on substrate with or without the corresponding enzyme. This would tend to indicate that some bacterial strains were capable of synthesizing their own enzymes and/or that the minimal media used supported bacterial growth to the extent that differences between enzyme and non enzyme supplemented substrates were minimal.

3.5.2 Bacterial growth in ethanol-extracted soybean and canola meals with or without enzyme supplementation

Addition of a multi-carbohydrase (containing xylanase, glucanase, cellulase, mannanase, pectinase and other NSP degrading activities), enzyme (Meng et al. 2005) to either soybean or canola based meal resulted in only moderate increases (approximately 0.1 to 0.5 log) in bacterial numbers. Interestingly, the largest increases were exhibited by LAB, namely *Lactobacillus brevis* and *Bifidobacterium pullorum*, and only when grown on canola meal.

The lack of a growth response by many bacteria, especially within 6 h, may indicate that the concentration of simple sugars produced following enzyme reaction was limiting. This observation appears to be further supported by the additional decrease in microbial growth during the ensuing 24 h. The main function for this commercial multi-carbohydrase enzyme was to reduce feed

viscosity in the chicken GIT. Therefore, although enzyme treatment of the feeds resulted in varying degrees of polysaccharide degradation, substrate limitation due to slow hydrolysis may have been responsible for the paucity of microbial growth. Meng *et al.* (2005) determined that the efficacy of this carbohydrase preparation in hydrolyzing soybean and canola meal NSPs, under the assay conditions used (pH 5.2, 45°C), averaged 26% and 36%, respectively. Differences in enzyme efficiency between the two meals coupled with a higher total NSP content in canola meal (17.9%) (Slominski and Campbell, 1990) compared to soybean meal (14.5%) (Huisman et al., 1998) may be why many of the test bacteria exhibited enhanced growth on enzyme-treated canola meal. Further, based on the component sugar profiles of both meals (Meng et. al., 2005) the glucose component of canola NSPs was approximately one-third higher compared to SBM. The higher level of this sugar could also have contributed to the difference in bacterial growth between the meals, assuming its higher availability as a noncomplex molecule.

3.5.3 Broiler chicken experiment

Various studies (Vahjen et al., 1998; Yasar and Forbes, 2000; Meng et al. 2005) have investigated the effects of exogenous enzyme supplementation on broiler performance; however, the results have often been inconsistent or inconclusive. In some cases the lack of a positive response may have been due to

variations in the content and complexity of NSPs in the feed ingredients and/or the nature (activity, specificity) of the added enzymes (Coon et al, 1990).

The microflora of the GI tract is complex, largely undefined and the number of species present is as high as 400 to 500 (Bedford and Apajalahti, 2001). Analyzing this complex environment makes it nearly impossible to enumerate all possible microorganisms using conventional techniques (Vahjen et al., 1998). Therefore, in order to assess possible changes in the microflora associated with the inclusion of enzyme into feeds, several specific groups and or microorganisms were chosen for study based on their close association with the GIT of poultry or potential to induce pathology (Stavric and Kornegay, 1995).

Overall, one of the more interesting findings of this study was that significant changes in the microbial population (ileum or caecum) were not observed between broilers maintained on barley supplemented with either glucanase A or glucanase B. Considering that the glucanase B preparation resulted in much higher liberation of glucose, it would be expected that microbial populations would also be higher. The lack of a positive response indicated that the enzyme, although capable of hydrolzing β-glucans was perhaps less efficient on a complex substrate like barley. Alternatively, the nutrient availability of the feed may have been sufficiently complete and any free sugars produced via enzyme hydrolysis did not generate higher microbial numbers (at least those that were specifically targeted).

The results of this study indicated that broilers maintained on corn or wheat based diets supplemented with a multi-carbohydrase enzyme system

contained significantly lower levels of *E. coli* especially in their caeca. In contrast, enterococci levels were not significantly affected. In addition, changes in the microbial profile of both microorganisms in the ileum were not readily apparent. Obviously, conditions for colonization and or growth by *E. coli* differ from those of the enterococci and one can only theorize the causes and major factors affecting host tissue response as well as influence of the physical and chemical properties of the digesta. For example, pH is well recognized to influence both growth and survival of microorganisms. The pH of the ileum and caecum reported by Sturkie (1976) was 6.4 and 5.7, respectively. Although variations in pH have been reported for both the ileum and caecum, a pH difference of 1 to 1.5 is possible and is likely to influence the composition and quantity of bacteria able to proliferate in these areas. Differences in pH normally result as a consequence of diet and age (Moran, 1982; Coon *et al.*, 1990). In addition and perhaps closely related to diet, the digestive actions of the host, including acid production by the stomach or bicarbonate secretion by the intestine have also been reported to affect pH (Vahjen *et al.*, 1998). The colon and caeca are anaerobic often having a redox potential less than -400 mV (Bedford and Apajalahti, 2001) and are major sites for microbial fermentation (Coon *et al.*, 1990). Volatile fatty acids (VFA) including acetic acid are often produced during fermentation of glucose via enzyme hydrolysis of starch. VFAs are antimicrobial and therefore can influence microbial types and numbers (Vahjen *et al.*, 1998). Changes in the concentration of bile salts within the GIT have also been linked to viscosity reduction via enzyme supplementation (Danicke *et al*, 1999).

Similar to the results of the present study, Jordan *et al.* (2001) noted that *E. coli* are always found in the GI tract of poultry, especially in the caeca. Riesenfeld *et al.* (1980) indicated that the majority of glucose absorption occurs in the duodenum of the small intestine and that 97% of glucose and starch is already absorbed before entering the ileum. This could indicate a substrate limitation for *E. coli* and enterococci in the ileum. The variation in microbial content of the caeca and ileum is also likely to affect the growth of *E. coli* and enterococci because individual existence of a microorganism depends upon complex interactions with other bacteria (Salanitro *et al.*, 1978).

In general, both aerobic and anaerobic spore levels in the ileum and caeca of broilers fed enzyme-supplemented diets increased. Additional increases in aerobic spore levels due to addition of probiotic were only observed in the caeca of broilers fed a corn-based diet. Anaerobic spore levels also increased due to addition of the probiotic. Although these increases would be expected based on the nature of the probiotic, it appears that growth or survival conditions in the caeca were less hospitable compared to the ileum. In part this may have been due to environmental differences including redox potential and/or pH (Moran, 1982).

The purpose of including a probiotic supplement in the diets was to determine if their use in concert with exogenous enzymes would provide an even greater health benefit to the animal, perhaps even a synergistic interaction. The probiotic powder contained a lyophilized suspension consisting of *Bacillus subtilis* and *Bacillus lichenformis* in a ratio of 4:1. Both microorganisms are mesophiles and facultative anaerobes and were present as spores. Ostensibly in

the spore state the bacteria would maintain longer viability and exhibit enhanced resistance to processing factors including heat. In addition, spores would be more resistant to the bile salts and stomach acids in the host animal following ingestion. Alexopoulos *et al.* (2004) used these bacteria as probiotics in swine and reported a decrease in morbidity. The authors stated that the exact mechanism for probiotic action remains unclear but may have been due to competitive exclusion. However, the authors further reported that the outcome of probiotic supplementation was sometimes inconsistent and that in many trials no positive effects, in terms of weight gain, were observed when fed to pigs.

The growth performance of the broiler chickens in terms of body weight gain and feed to weight gain ratio was affected by dietary enzyme supplementation. Enzyme supplementation was shown to increase NSP digestibility in both canola meal (Slominski and Campbell, 1990; Simbaya *et al.*, 1996; Kocher *et al.*, 2000) and corn-SBM diets (Marsman *et al.*, 1997; Douglas *et al.*, 2000; Kocher *et al.*, 2000), and growth performance was also positively affected. Yasar and Forbes (2000) also performed a broiler trial using a diet high in wheat with and without addition of exogenous enzymes (xylanase, glucanase and protease). A significant decrease in digesta viscosity was observed with enzyme supplementation, however, the authors concluded that the enzyme did not significantly affect food intake or live weight gain. The addition of enzyme and or probiotic to diets used in this study affected the population dynamics of some bacterial groups. This may have had some effect on growth performance.

The addition of probiotics to feeds has been shown to be beneficial in numerous animals with respect to growth performance and overall health, however, an equal number of studies have concluded that there were no benefits following use. Numerous factors are known to influence the efficacy of probiotics in animal husbandry. One of the most important factors is the general health of the animal. Animals which are otherwise healthy and relatively free from stress (such as those used in this study) do not generally benefit from the addition of probiotics to their diets (Bedford and Apajalahti, 2001). The lack of a large growth response by broiler chickens in this study and perhaps in previous studies may also have been due to the relative crude nature of the enzyme preparations used (Bedford and Classen, 1993). Overall, due to the complex nature of the NSP fractions, it would be expected that enzyme preparations with a number of activities (multi-carbohydrase) would be more effective compared to single enzyme preparations. However, even multi-carbohydrase enzymes may prove ineffective unless the combination, concentration, mode of action and activity are matched to the potential NSPs that are to be hydrolyzed. Researchers including Meng *et al.* (2005) supported the idea that a combination of carbohydrases is more likely to be effective in poultry and will increase broiler performance. Multi-carbohydrases have the ability to react with a wider range of feed ingredients, producing a greater array of metabolizable compounds. Cocktail preparations would also be more versatile since they could be used with a variety of poultry species of different ages. Individual pure enzymes, although having a

more targeted function and predictable results, may not hydrolyze sufficient substrate to cause any changes in gut bacteria.

Furthermore, the ability to determine which enzymes promote the growth of beneficial GIT bacteria will also benefit the producer by improving the natural health of the animal and possibly decrease the occurrence of disease. In contrast, the possible growth promotion of certain pathogenic bacteria by the addition of exogenous enzymes must also be considered. If enzymes contribute to increased levels of pathogenic bacteria, then this could increase the health risk to the animals and therefore cause increased cost to the producer and potential health dangers to the consumer.

4.0 CONCLUSION

Exogenous enzymes can reduce intestinal viscosity of poultry which can improve feed digestibility and health. Other effects, such as alteration of the microbial gut population appear to be less clear. In addition, the inclusion of enzymes in feeds in the present study did not appear to select for any specific microbial species. Therefore additional feed trials are highly recommended. It is possible that significant changes in the growth of various gut bacteria would become more noticeable by use of microbiologically-challenged birds since the ones used in the present study would be considered healthy. Future feeding trials evaluating the effects of enzymes in feeds should consider employing birds that would be initially challenged with pathogens such as *E. coli* or salmonellae (in the drinking water or by gavage).

In conclusion the salient results of the present study are:

1. A positive correlation existed between enzymes that released relative high levels of glucose (monosaccharides) and microbial growth.
2. Probiotic addition did not consistently alter the bacterial growth for any of the microorganisms tested.
3. Exogenous enzyme addition consistently resulted in higher bird weight gain and lower feed to gain ratios.
4. Addition of the enzymes investigated (*in vitro and in vivo*) did not select for any particular group of bacteria.

REFERENCES

Acamovic, T. 2001. Commercial application of enzyme technology for poultry production. World Poult. Sci. J. 57:225-242.

Alexopoulos, C., Georgoulakis, I. E., Tzivara, A., Kyriakis, C.S., Govaris, A. and Kyriakis, S.C. 2004. Field evaluation of the effect of a probiotic-containing *Bacillus licheniformis* and *Bacillus subtilis* spores on the health status, performance, and carcass quality of grower and finisher pigs. J. Vet. Med. 51:306-312.

Annison, G. 1991. Relationship between the levels of soluble nonstarch polysaccharides and the apparent metabolisable energy of wheat assayed in broiler chickens. J. Agricult. Food Chem. 29:1252-1256.

Apajalahti, J. 1998. Nutritional effects on the microflora of the GI tract. Western Nutritional Conference. Saskatoon, Saskatchewan. Sept 1998. 60-68.

Apajalahti, J. and Bedford, M.R. 1999. Improve bird performance by feeding its microflora. World Poult. Sci. J. 15:20-23.

Aspinall, G.O. 1982. Analysis of polysaccharides. In: *Food Carbohydrates*. Lineback, D.R. and Inglett, G.E. ed. Westport, CT. pp. 357-374.

Austin, S.C., Wiseman, J. and Chesson, A. 1999. Influence of non-starch polysaccharides structure on the metabolisable energy of UK wheat fed to poultry. J. Cereal Sci. 29:77-88.

Bedford, M.R. and Classen, H.L. 1993. An *in vitro* assay for prediction of broiler intestinal viscosity and growth when fed rye-based diets in the presence of exogenous enzymes. Poult. Sci. 72:137-143.

Bedford, M.R. and Morgan, A.J. 1996. The use of enzymes in poultry diets. World Poult. Sci. J. 52:61-68.

Bedford, M.R. 2000. Exogenous enzymes in monogastric nutrition – their current value and future benefits. Anim. Feed Sci. Technol. 86:1-13.

Bedford, M.R. and Apajalahti, J. 2001. Microbial interactions in the response to exogenous enzyme utilization. *Enzymes in Farm Animal Nutrition*. Bedford, M.R. and Partridge, G.G. ed. CAB International. pp. 299-314.

Blaut, M. 2002. Relationship of probiotics and food to intestinal microflora. Eur. J. Nutr. 41:11-16.

Brufau, J., Francesch, M. and Perez-Vendrell, A.M. 2001. NSP-degrading enzymes on poultry feeding: recent developments and future prospects. 13th European Symposium of Poultry Nutrition. 17-21.

Campbell, G.L., Rossnagel, B.F., Classen, H.L. and Thacker, P.A. 1989. Genotypic and environmental differences in extract viscosity of barley and their relationship to its nutritive value for broiler chickens. Anim. Feed Sci. Technol. 26:221-230.

Chesson, A. 2001. Non-starch polysaccharide degrading enzymes in poultry diets: influence of ingredients on the selection of activities. World Poult. Sci. J. 57:251-262.

Choct, M. 2001. Enzyme supplementation of poultry diets based on viscous cereals. Pages 145-159 in: *Enzymes in Farm Animal Nutrition.* Bedford, M.R. and Partridge, G.G. ed. CAB International.

Choct, M. and Annison, G. 1992. Anti-nutritive effect of wheat pentosans in broiler chickens: roles of viscosity and gut microflora. Br. Poult. Sci. 33:821-834.

Choct, M., Annison, G. and Trimble, R.P. 1993. Extract viscosity as a predictor of the nutritive quality of wheat in poultry. Proceedings of the Australian Poultry Science Symposium. 5:78-83.

Choct, M. and Kocher, A. 2000. Excreta viscosity as an indicator of microbial enzyme activity in the hindgut and as a predictor of between-bird variation in AME in broilers. *Proceedings of the Australian Poultry Science Symposium.* 12:211.

Coon, C.N., Leske, K.L., Akavanichan, O. and Cheng, T.K. 1990. Effect of oligosaccharide-free soybean meal on true metabolisable energy and fiber digestion in adult roosters. Poult. Sci. 69:787-793.

Danicke, S., Simon, O., Jeroch, H. and Bedford, M.R. 1999. Interactions between dietary fat type and exogenous enzyme supplementation of broiler diets based on maize, wheat, triticale or barley. J. Anim. Feed Sci. 8:467-483.

Douglas, M.W., Parsons, C.M. and Bedford, M.R. 2000. Effect of various soybean meal sources and avizyme on chick growth performance and ileal digestible energy. J. Appl. Poult. Res. 9:74-80.

Englyst, H.N. and Cummings, J.H. 1984. Simplified method for measurement of total non-starch polysaccharides by gas-liquid chromatography of constituent sugars as alditol acetates. Analyst. 109:937-942.

Francesch, M., Perez-Moya, S., Badiola, I. and Brufau, J. 1999. Effects of cereal and feed enzyme on digesta viscosity and bacterial count in broiler chickens. In: Proceedings of the 12th European Symposium on Poultry Nutrition. pp. 242-245. WPSA-Dutch Branch, Veldhoven.

Fuller, R. 1989. Probiotics in man and animals. J. Appl. Bacteriol. 66:365-378.

Hesselman, K. and Aman, P. 1986. The effect of β-glucanase on the utilization of starch and nitrogen by broiler chickens fed on barley of low or high viscosity. Anim. Feed Sci. Technol. 15:83-93.

Huisman, M.M.H., Schols, H.A. and Voragen, A.G.J. 1998. Cell wall polysaccharides from soybean (*Glycine max*) meal. Isolation and characterization. Carbohydr. Polym. 37:87-95.

Inborr, J. and Bedford, M.R. 1994. Stability of feed enzymes to steam pelleting during feed processing. Anim. Feed Sci. Technol. 46:179-196.

Jin, L.Z., Ho, Y.W., Abdullah, N. and Jalaludin, S. 1997. Probiotics in poultry: modes of action. World Poult. Sci. J. 53:351-363.

Jordan, F., Pattison, M., Alexander, D. and Farager, T. 2001. Poultry Diseases. Harcourt Publishers. London.

Kocher, A., Choct, M., Porter, M.D., and Broz, J. 2000. The effects of enzyme addition to broiler diets containing high concentration of canola and sunflower meal. Poult. Sci. 79:1767-1774.

Koneman, E. W. 1988. Color atlas and textbook of diagnostic microbiology. 3rd edition. J.B. Lippincott Co., Philadelphia. p. 94.

Malathi, V. and Devegowda, G. 2001. *In vitro* evaluation of nonstarch polysaccharide digestibility of feed ingredients by enzymes. Poult. Sci. 80:302-305.

Marquardt, R.R. 1997. Enzyme enhancement of the nutritional value of cereals: role of viscous, water-soluble, nonstarch polysaccharides in chick performance. Pages 5-16 in: *Enzymes in Poultry and Swine Nutrition.* Marquardt, R.R. and Han, Z. ed. International Development Research Centre. Ottawa, Ontario, Canada.

Marquardt, R.R. and Bedford, M.R. 2001. Future horizons. Pages 389-398 in: *Enzymes in Farm Animal Nutrition.* Bedford, M.R. and Partridge, G.G. ed. CAB International.

Marsman, G.J.P., Gruppen, H., van der Poel, A.F.B., Kwakkel, A.F., Verstegen, M.W.A. and Vogagen, A.G.J. 1997. The effect of thermal processing and enzyme treatments of soybean meal on growth performance, ileal nutrient digestibilities, and chime characteristics in broiler chicks. Poult. Sci. 76:864-872.

Meng, X., Slominski, B.A., Nyachoti, C.M., Campbell, L.D. and Guenter, W. 2005. Degradation of cell wall polysaccharides by combinations of carbohydrase enzymes and

their effects on nutrient utilization and broiler chicken performance. Poult. Sci. 84:37-47.

Merck Index. 2004. 13th Edition. Merck & Co., Inc. Whitehouse Station, NJ, USA.

Moran, E.T. 1982. Comparative nutrition of fowl and swine: The gastrointestinal systems. E.T. Moran Jr. Canada. pp. 45-52.

Nurmi, E. and Rantala, M. 1973. New aspects of *Salmonella* infection in broiler production. Nature. 241:210-211.

Pettey, L.A., Carter, S.D., Senne, B.W. and Shriver, J.A. 2002. Effects of β-mannanase addition to corn-soybean meal diets on growth performance, carcass traits, and nutrient digestibility of weanling and growing-finishing pigs. J. Anim. Sci. 80:1012-1019.

Philip, J.S., Gilbert, H.J. and Smithard, R.R. 1995. Growth, viscosity and beta-glucanase activity of intestinal fluid in broiler chickens fed on barley-based diets with or without exogenous beta-glucanase. Br. Poult. Sci. 36:599-603.

Risesenfeld, G., Sklan, D., Bar, A., Eisner, U. and Hurwitz, S. 1980. Glucose absorption and starch digestion in the intestine of the chicken. J. Nutr. 110-117.

Rotter, B.A., Marquardt, R.R., Guenter, W., Biliaderis, C., and Newman, C.W. 1989. In vitro viscosity measurements of barley extracts as predictors of growth responses in chicks fed barley-based diets supplemented with a fungal enzyme preparation. Can. J. Anim. Sci. 69:431-439.

Salanitro, J.P., Blake, I.G., Muirhead, P.A., Maglio, M. and Goodman, J.R. 1978. Bacteria isolated from the duodenum, ileum, caecum of young chicks. Appl. Environ. Microbiol. 35:782.

Schutte, J.B. and Langhout, D.J. 1999. Influence of the intestinal microflora on health and performance of broiler chicks. Proceedings of the WPSA Spring Meeting, Scarborough, 22-24 March. pp. 57-58.

Scott, R.W. 1979. Colorimetric determination of hexuronic acids in plant materials. Anal. Chem. 51:936-941.

Sheppy, C. 2001. The current feed enzyme market and likely trends. Pages 1-10 in: *Enzymes in Farm Animal Nutrition*. Bedford, M.R. and Partridge, G.G. ed. CAB International.

Silva, S.S.P. and Smithard, R.R. 2002. Effect of enzyme supplementation of a rye-based diet on xylanase activity in the small intestine of broilers, on intestinal crypt cell proliferation and on nutrient digestibility and growth performance of the birds. Br. Poult. Sci. 43:274-282.

Simbaya, J.B., Slominski, B.A., Guenter, W., Morgan, A. and Campbell L. D. 1996. The effects of protease and carbohydrase supplementation on the nutritive value of canola meal for poultry: in vitro and in vivo studies. Anim. Feed Sci. Technol. 61:219-234.

Slominski, B.A. and Campbell, L.D. 1990. Non-starch polysaccharides of canola meal: quantification, digestibility in poultry and potential benefits of dietary enzyme supplementation. J. Sci. Food Agric. 53:175-184.

Slominski, B.A., Gdala, D., Boros, L.D., Campbell, L.D., Guenter, W. and Jones, O. 2000. Variability in chemical and nutritive composition of Canadian wheat and the potential for its minimization by enzyme use. In Proceedings of the XXI World Poultry Congress, Montreal, Canada.

Stavric, S. and D'Aoust, J.Y. 1993. Undefined and defined bacterial populations for the competitive exclusion of *Salmonella* in poultry – A review. J. Food Protect. 48:173-180.

Stavric, S. and Kornegay, E.T. 1995. Microbial probiotics for pigs and poultry. Pages 205-239 in: *Biotechnology in Animal Feeds and Animal Feeding*. Wallace, R.J. and Chesson, A. ed. VCH Publishing. Weinheim, Germany.

Sturkie, P.D. 1976. *Avian Physiology*. Springer-Verlag., New York. pp 270-280.

Vahjen, W., Glaser, K., Schafer, K. and Simon, O. 1998. Influence of xylanase-supplemented feed on the development of selected bacterial group in the intestinal tract of broiler chicks. J. Agric. Sci. 130:489-500.

Vranjes, M.V., Pfirter, M.P. and Wenk, C. 1994. Influence of processing treatment and type of cereal on the effect of dietary enzymes in broiler diets. Anim. Feed Sci. Technol. 46:261-270.

Vranjes, M.V. and Wenk, C. 1995. Influence of dietary enzyme complex on the performances of broilers fed on diets with and without antibiotic supplementation. Br. Poult. Sci. 36:265-275.

Yasar, S. and Forbes, J.M. 2000. Enzyme supplementation of dry and wet wheat-based feeds for broiler chickens: performance and gut responses. Br. J. Nutr. 84:297-307.

Zhang, Z., Marquardt, R.R., Wang, G., Guenter, W., Crow, G.H., Han, Z. and Bedford, M.R. 1996. A simple model for predicting the response of chicks to dietary enzyme supplementation. J. Anim. Sci. 74:394-402.

Appendix Table 1. Bacterial growth (change in absorbance) in basal medium with and without enzyme addition in various substrates. Values are means of triplicate determinations.

Organism	Time (h)	Absorbance at 340 nm									
		β-glucan		β-glucan		Xylan		Raffinose		Guar Gum	
		control	BBG 10,000	control	Biocellulase AZ	control	enzyme	control	enzyme	control	enzyme
Enterococcus faecalis	2	-0.004	0.030	0.010	0.024	-0.008	0.021	-0.012	0.046	0.005	0.023
	4	-0.037	0.029	0.005	0.285	-0.043	-0.002	-0.024	0.308	0.052	0.012
	6	-0.015	0.080	-0.005	0.325	-0.040	-0.004	-0.051	0.280	-0.005	0.042
	24	-0.086	0.097	-0.004	0.313	0.001	-0.013	0.120	0.251	0.019	0.150
Enterococcus gallinarum	2	0.016	-0.001	-0.012	-0.010	-0.006	0.001	-0.006	0.040	0.003	0.026
	4	0.002	0.009	-0.013	0.033	-0.036	0.023	0.008	0.226	-0.014	0.096
	6	-0.011	0.105	-0.033	0.257	-0.035	0.044	0.048	0.317	0.013	0.179
	24	-0.096	0.202	-0.033	0.121	-0.020	-0.015	0.056	0.029	0.014	0.230
Lactobacillus acidophilus	2	0.001	0.004	0.009	-0.016	0.002	0.002	-0.002	0.004	-0.028	0.017
	4	0.004	0.012	0.001	0.010	0.007	0.016	0.009	0.015	0.002	0.057
	6	0.005	0.022	-0.004	0.014	-0.006	-0.026	0.004	-0.003	-0.002	0.036
	24	0.011	0.045	0.018	0.035	-0.014	0.000	-0.005	0.023	0.014	0.202
Lactobacillus brevis	2	0.004	0.009	-0.013	0.001	0.003	0.015	0.010	0.005	0.009	0.053
	4	0.011	0.023	-0.018	0.093	0.000	0.015	-0.002	0.115	0.005	0.151
	6	0.013	0.065	-0.038	0.237	0.006	0.018	-0.015	0.414	0.017	0.216
	24	-0.002	0.149	-0.001	0.359	0.016	0.034	0.007	0.371	0.015	0.266

Appendix Table 1 continued.

Organism	Time (h)	Absorbance at 340 nm									
		β-glucan		β-glucan		Xylan		Raffinose		Guar Gum	
		control	BBG 10,000	control	Biocellulase AZ	control	enzyme	control	enzyme	control	enzyme
Lactobacillus rhamnosis	2	0.002	0.004	0.014	0.035	0.024	0.021	0.015	0.010	0.030	0.035
	4	0.003	0.040	0.004	0.052	-0.025	0.000	0.008	0.017	-0.026	-0.026
	6	0.011	0.039	0.003	0.098	-0.019	-0.022	0.017	0.081	0.017	0.050
	24	0.040	0.050	-0.014	0.170	0.076	0.086	0.027	0.276	0.129	0.200
Escherichia coli	2	0.006	-0.008	-0.003	0.112	0.006	0.021	0.011	0.017	-0.031	0.040
	4	-0.015	0.008	-0.001	0.187	0.029	0.023	-0.009	0.190	-0.046	0.117
	6	-0.003	0.029	-0.004	0.197	-0.008	0.085	0.001	0.292	-0.034	0.131
	24	-0.046	-0.124	-0.046	0.135	-0.038	-0.033	-0.036	-0.011	-0.087	0.023
Clostridium perfringens	2	0.005	0.034	0.002	0.034	-0.011	-0.006	0.009	0.010	0.001	-0.004
	4	0.007	0.044	0.003	0.056	0.002	0.003	0.022	0.248	0.025	0.028
	6	0.014	0.067	0.009	0.090	-0.004	0.023	0.007	0.272	0.018	0.033
	24	0.022	0.118	0.022	0.177	-0.010	-0.011	0.011	0.212	0.036	0.048
Listeria monocytogenes	2	-0.018	0.008	-0.012	0.053	-0.007	-0.006	0.002	0.062	-0.013	-0.008
	4	0.003	0.036	-0.008	0.151	0.001	-0.007	0.006	0.258	-0.024	0.009
	6	-0.009	0.069	-0.002	0.156	0.001	-0.009	-0.004	0.200	-0.028	0.076
	24	0.011	-0.062	0.005	0.208	-0.024	-0.005	0.017	0.215	0.018	0.018

Appendix Table 1 continued.

Organism	Time (h)	Absorbance at 340 nm									
		β-glucan		β-glucan		Xylan		Raffinose		Guar Gum	
		control	BBG 10,000	control	Biocellulase AZ	control	enzyme	control	enzyme	control	enzyme
Salmonella Typhimurium	2	0.015	0.022	0.003	0.072	-0.022	-0.006	0.012	0.049	-0.025	0.015
	4	-0.004	0.013	0.003	0.164	-0.019	0.027	0.002	0.224	-0.009	0.100
	6	-0.015	0.025	-0.004	0.015	0.015	-0.017	0.006	0.178	-0.048	0.066
	24	-0.076	0.009	0.009	0.182	0.109	0.178	-0.001	0.233	0.045	0.121
Salmonella pullorum	2	0.008	0.025	0.004	0.085	0.009	0.014	-0.005	0.083	0.006	0.028
	4	0.015	0.024	0.011	0.175	0.013	-0.016	-0.013	0.207	-0.001	0.038
	6	0.006	0.038	-0.001	0.174	0.027	-0.012	0.029	0.234	-0.021	0.009
	24	0.042	0.075	-0.015	0.229	0.094	0.030	0.056	0.168	0.001	0.068
Bifidobacterium animalis	2	-0.010	0.022	-0.009	0.025	-0.025	-0.001	-0.019	0.030	-0.030	0.004
	4	-0.011	0.022	-0.005	0.183	0.004	0.012	0.003	0.210	-0.012	0.120
	6	0.000	0.030	-0.006	0.196	-0.003	0.026	0.000	0.216	-0.001	0.136
	24	0.003	0.089	0.001	0.184	0.023	0.019	0.021	0.174	0.016	0.166
Bifidobacterium pullorum	24	-0.014	0.047	0.010	0.046	-0.007	-0.021	0.020	0.027	-0.017	0.127
	48	0.000	0.056	0.033	0.145	0.051	0.044	0.067	0.028	-0.014	0.155
	72	0.004	0.033	0.021	0.140	0.066	0.066	0.094	0.062	0.014	0.138

Appendix Table 1 continued.

Organism	Time (h)	Absorbance at 340 nm									
		β-glucan		β -glucan		Xylan		Raffinose		Guar Gum	
		control	BBG 10,000	control	Biocellulase AZ	control	enzyme	control	enzyme	control	enzyme
Bifidobacterium gallinarum	24	0.007	0.071	-0.002	0.113	-0.003	0.021	0.001	0.119	0.013	0.078
	48	0.014	0.041	0.027	0.152	-0.003	0.077	0.012	0.143	0.008	0.058
	72	0.033	0.022	0.016	0.181	-0.012	0.115	-0.012	0.111	-0.021	0.038
Campylobacter jejuni	24	0.003	0.034	0.017	0.009	0.015	-0.007	0.024	0.021	0.034	0.121
	48	0.032	0.040	0.025	0.142	0.022	0.105	0.025	0.055	0.011	0.129
	72	0.040	0.098	0.022	0.212	0.024	0.076	0.031	0.108	-0.015	-0.034

Appendix Table 2. Microbiological results of chicken trial for treatment 1[1].

Sample	Log_{10} CFU/g							
	E. coli caeca	*E. coli* ileum	enterococci caeca	enterococci ileum	aerobic spore caeca	aerobic spore ileum	anaerobic spore caeca	anaerobic spore ileum
1	8.08	4.24	5.78	3.95	4.26	3.23	4.15	3.32
2	8.45	4.43	5.46	4.23	4.00	3.30	3.95	3.45
3	7.29	3.97	4.72	3.82	3.78	3.36	3.78	3.75
4	7.26	4.56	5.22	3.72	5.15	4.23	3.95	3.95
5	6.48	4.67	6.21	4.96	3.85	4.79	3.78	3.61
6	7.08	5.06	6.08	3.74	3.90	3.34	3.90	3.78
7	7.18	4.98	4.15	4.23	3.95	3.11	3.85	4.03
8	7.56	5.35	4.85	4.12	4.08	5.18	3.78	4.49
9	6.85	5.23	5.06	3.62	3.85	3.67	3.78	3.45
Average	7.36	4.72	5.28	4.04	4.09	3.80	3.88	3.76
Std Dev.	0.60	0.47	0.67	0.41	0.42	0.75	0.13	0.36

[1] Wheat/soybean/canola meal - control (no additive).

Appendix Table 3. Microbiological results of chicken trial for treatment 2[1].

Sample	Log_{10} CFU/g							
	E. coli caeca	*E. coli* ileum	enterococci caeca	enterococci ileum	aerobic spore caeca	aerobic spore ileum	anaerobic spore caeca	anaerobic spore ileum
1	7.63	3.76	5.50	4.08	4.15	3.65	6.35	3.42
2	7.02	4.45	5.85	4.01	6.90	4.46	5.97	4.00
3	7.40	4.41	6.70	4.62	6.88	3.49	5.26	3.26
4	6.79	4.98	4.92	4.50	6.56	3.81	5.90	3.98
5	6.83	5.92	5.29	4.06	6.12	3.66	7.14	4.16
6	6.48	4.24	4.40	3.79	6.35	4.86	5.88	4.08
7	6.87	4.92	6.36	4.12	6.12	3.83	6.36	3.79
8	6.60	5.07	6.48	3.94	5.33	4.24	3.78	4.50
9	6.26	4.74	4.95	3.81	6.01	3.98	3.78	4.28
Average	6.88	4.72	5.61	4.10	6.05	4.00	5.60	3.94
Std. Dev.	0.43	0.61	0.80	0.29	0.86	0.44	1.15	0.40

[1] Wheat/soybean/canola meal + xylanase.

Appendix Table 4. Microbiological results of chicken trial for treatment 3[1].

Sample	Log_{10} CFU/g							
	E. coli caeca	*E. coli* ileum	enterococci caeca	enterococci ileum	aerobic spore caeca	aerobic spore ileum	anaerobic spore caeca	anaerobic spore ileum
1	6.22	5.32	4.64	4.71	6.14	3.23	5.05	5.29
2	6.26	5.39	5.43	3.42	6.22	3.62	5.69	5.32
3	5.97	5.43	4.59	4.84	6.46	3.73	5.98	5.35
4	6.11	5.31	4.52	3.50	5.43	3.64	5.17	6.56
5	6.02	5.03	5.40	4.97	6.71	4.23	5.00	5.29
6	6.48	2.84	4.57	2.54	5.64	3.80	6.60	5.34
7	6.26	4.30	4.32	3.87	5.33	5.07	5.42	6.34
8	6.70	5.90	5.61	4.00	5.62	4.93	5.58	5.17
9	6.26	5.91	5.61	4.70	6.79	5.07	5.52	5.19
Average	6.25	5.05	4.97	4.06	6.04	4.15	5.56	5.54
Std. Dev.	0.23	0.96	0.53	0.82	0.55	0.71	0.50	0.52

[1] Wheat/soybean/canola meal + xylanase + probiotic (*Bacillus subtilis* and *Bacillus licheniformis*).

Appendix Table 5. Microbiological results of chicken trial for treatment 4[1].

Sample	Log_{10} CFU/g							
	E. coli caeca	*E. coli* ileum	enterococci caeca	enterococci ileum	aerobic spore caeca	aerobic spore ileum	anaerobic spore caeca	anaerobic spore ileum
1	6.78	5.38	5.98	3.78	3.95	3.88	4.00	3.72
2	5.97	5.47	6.15	3.41	4.68	4.07	3.85	3.34
3	7.34	5.15	5.97	3.73	4.72	4.18	4.00	3.92
4	6.36	5.35	5.79	3.43	4.46	4.10	4.15	4.01
5	6.48	4.46	4.77	4.85	4.34	3.69	4.32	4.05
6	6.70	4.86	5.31	4.79	5.52	3.04	4.79	3.04
7	6.85	3.20	6.15	4.35	4.26	3.28	4.23	3.15
8	6.24	4.60	5.57	4.21	5.68	3.69	4.95	2.78
9	6.48	5.16	5.36	4.13	4.04	2.78	4.00	3.32
Average	6.58	4.85	5.67	4.07	4.63	3.63	4.25	3.48
Std. Dev.	0.40	0.71	0.46	0.53	0.61	0.50	0.38	0.46

[1] Barley/soybean/canola meal – control (no additive).

Appendix Table 6. Microbiological results of chicken trial for treatment 5[1].

Sample	Log_{10} CFU/g							
	E. coli caeca	*E. coli* ileum	enterococci caeca	enterococci ileum	aerobic spore caeca	aerobic spore ileum	anaerobic spore caeca	anaerobic spore ileum
1	8.32	5.45	6.27	4.61	6.55	3.00	5.98	3.15
2	7.59	3.70	6.81	4.12	6.24	6.34	5.77	4.05
3	8.07	5.32	7.99	5.50	6.11	4.38	6.13	3.58
4	6.60	4.51	6.88	1.85	4.48	6.13	4.00	3.56
5	6.48	4.94	6.71	4.90	6.98	6.14	5.73	2.95
6	6.78	5.91	7.12	4.13	5.71	6.15	5.80	4.10
7	6.70	5.17	6.57	3.20	4.40	5.15	4.49	3.60
8	6.90	5.03	6.98	3.73	6.34	5.87	5.89	3.08
9	6.70	5.28	6.49	4.71	6.70	3.70	4.04	3.20
Average	7.13	5.04	6.87	4.08	5.95	5.20	5.32	3.47
Std. Dev.	0.69	0.63	0.49	1.08	0.93	1.23	0.87	0.41

[1] Barley/soybean/canola meal + Glucanase A.

Appendix Table 7. Microbiological results of chicken trial for treatment 6[1].

Sample	Log_{10} CFU/g							
	E. coli caeca	*E. coli* Ileum	enterococci caeca	enterococci ileum	aerobic spore caeca	aerobic spore ileum	anaerobic spore caeca	anaerobic spore ileum
1	7.53	3.94	6.98	2.85	6.46	5.38	5.78	3.74
2	8.69	5.44	6.11	5.65	6.29	4.72	5.07	4.56
3	7.73	4.30	6.79	3.73	5.85	5.73	5.31	3.48
4	6.60	3.63	5.98	5.85	6.79	5.96	5.13	3.80
5	7.53	6.08	6.88	4.64	4.74	3.78	4.42	4.08
6	7.59	4.64	6.26	2.90	4.89	6.25	4.32	3.32
7	6.85	4.00	6.88	3.83	6.47	5.96	5.96	3.30
8	7.00	5.35	6.80	4.22	6.37	3.26	6.13	4.23
9	6.48	5.32	6.98	3.79	6.34	6.13	5.75	3.67
Average	7.33	4.75	6.63	4.16	6.02	5.24	5.32	3.80
Std. Dev.	0.69	0.84	0.40	1.06	0.73	1.09	0.65	0.42

[1] Barley/soybean/canola meal + Glucanase B.

Appendix Table 8. Microbiological results of chicken trial for treatment 7[1].

Sample	Log_{10} CFU/g							
	E. coli caeca	*E. coli* ileum	enterococci caeca	enterococci ileum	aerobic spore caeca	aerobic spore ileum	anaerobic spore caeca	anaerobic spore ileum
1	7.09	4.22	6.79	2.78	5.21	5.32	5.07	5.25
2	7.30	5.07	7.30	6.09	6.33	5.34	5.06	5.17
3	7.36	4.94	6.34	4.81	6.37	5.50	5.42	5.18
4	7.75	5.67	6.89	6.52	6.80	5.16	5.24	5.20
5	7.91	5.72	6.80	4.07	5.51	5.47	5.63	5.56
6	7.43	5.43	6.93	3.11	5.40	5.12	5.77	5.05
7	7.28	4.23	6.33	0.00	5.23	5.24	5.16	5.34
8	6.90	4.79	7.71	5.87	6.34	4.71	4.98	5.62
9	7.45	5.43	6.74	4.47	6.16	5.01	4.96	5.87
Average	7.39	5.05	6.87	4.19	5.93	5.21	5.25	5.36
Std. Dev.	0.31	0.56	0.43	2.03	0.59	0.25	0.29	0.27

[1] Barley/soybean/canola meal + Glucanase B + probiotic (*Bacillus subtilis* and *Bacillus licheniformis*).

Appendix Table 9. Microbiological results of chicken trial for treatment 8[1].

Sample	Log_{10} CFU/g							
	E. coli caeca	*E. coli* ileum	enterococci caeca	enterococci ileum	aerobic spore caeca	aerobic spore ileum	anaerobic spore caeca	anaerobic spore ileum
1	8.14	5.44	6.68	4.84	5.09	3.82	5.12	4.47
2	7.23	5.26	5.81	4.29	4.52	3.18	4.75	3.59
3	8.20	5.44	6.08	4.90	4.99	2.78	5.01	3.76
4	6.48	5.94	5.20	4.24	3.90	3.46	4.04	3.95
5	6.60	5.75	4.34	4.19	3.26	3.40	4.43	4.22
6	6.48	4.35	6.32	4.22	3.95	3.79	4.15	3.95
7	6.90	5.96	6.46	4.00	4.68	4.56	4.45	3.04
8	6.48	4.64	5.85	3.23	4.72	4.52	4.04	3.08
9	7.71	5.53	6.42	4.22	4.56	3.32	3.85	3.00
Average	7.14	5.37	5.91	4.24	4.41	3.65	4.43	3.67
Std. Dev.	0.72	0.55	0.74	0.48	0.59	0.59	0.45	0.54

[1] Corn/soybean/canola meal – control (no additive).

Appendix Table 10. Microbiological results of chicken trial for treatment 9[1].

Sample	Log_{10} CFU/g							
	E. coli caeca	*E. coli* ileum	enterococci caeca	enterococci ileum	aerobic spore caeca	aerobic spore ileum	anaerobic spore caeca	anaerobic spore ileum
1	5.86	5.47	6.00	3.98	6.37	4.74	6.12	4.73
2	6.13	5.39	6.26	3.59	6.19	4.45	6.31	4.45
3	6.63	5.30	6.01	4.92	6.25	5.90	5.89	5.09
4	5.37	4.11	6.05	4.24	4.76	5.27	4.73	3.23
5	5.30	5.05	5.88	4.23	4.15	5.15	4.20	3.23
6	6.00	4.78	4.40	3.89	6.92	4.89	4.23	4.05
7	6.90	5.84	6.45	4.15	5.45	6.13	6.23	3.20
8	6.36	4.24	6.82	4.84	5.42	4.62	4.96	2.78
9	6.25	6.45	6.56	4.21	5.97	3.15	4.45	3.20
Average	6.09	5.18	6.05	4.23	5.72	4.92	5.24	3.77
Std. Dev.	0.53	0.74	0.69	0.43	0.87	0.87	0.89	0.82

[1] Corn/soybean/canola meal + multi-carbohydrase enzyme (cocktail of cell wall degrading activities (1000 U xylanase, 400 U glucanase, 1000 U pectinase, 120 U cellulose, 280 U mannanase and 180 U galactanase).

Appendix Table 11. Microbiological results of chicken trial for treatment 10[1].

Sample	Log_{10} CFU/g							
	E. coli caeca	*E. coli* Ileum	enterococci caeca	enterococci ileum	aerobic spore caeca	aerobic spore ileum	anaerobic spore caeca	anaerobic spore ileum
1	6.48	3.23	5.59	4.50	6.78	4.69	6.90	5.06
2	5.74	3.24	6.34	5.50	6.90	5.00	6.85	5.88
3	6.16	3.26	5.85	5.20	7.23	4.88	3.90	5.37
4	5.71	5.24	6.98	4.62	5.15	4.68	5.52	6.14
5	5.75	3.88	5.08	4.51	7.13	5.22	5.70	5.14
6	5.63	4.23	4.48	5.22	7.11	5.03	6.88	5.20
7	6.60	6.01	5.58	4.67	5.17	4.96	6.34	4.87
8	6.48	4.33	4.46	0.00	6.90	5.24	6.13	4.89
9	5.86	3.63	6.20	3.61	6.85	5.09	6.35	5.22
Average	6.05	4.12	5.62	4.20	6.58	4.98	6.06	5.31
Std. Dev.	0.39	0.97	0.84	1.67	0.82	0.20	0.95	0.43

[1] Corn/soybean/canola meal + multi-carbohydrase enzyme (cocktail of cell wall degrading activities (1000 U xylanase, 400 U glucanase, 1000 U pectinase, 120 U cellulose, 280 U mannanase and 180 U galactanase) + probiotic (*Bacillus subtilis* and *Bacillus licheniformis*).

Appendix Table 12. Broiler chicken trial.

Diet	Pen	Body weight gain g/bird	Feed intake g/bird	Feed to gain ratio
1	6	352.7	499.0	1.415
	14	362.2	503.8	1.391
	16	384.2	540.0	1.406
	40	348.6	497.1	1.426
	61	398.5	601.0	1.508
	67	405.2	562.1	1.387
	80	371.6	512.8	1.380
2	12	400.2	544.4	1.360
	25	380.3	500.8	1.317
	26	404.6	526.6	1.302
	34	388.7	522.3	1.344
	41	399.3	536.3	1.343
	51	393.5	526.8	1.339
	69	405.9	531.6	1.310
	78	403.5	532.7	1.320
3	1	388.5	524.5	1.350
	7	402.8	532.5	1.322
	18	417.2	562.7	1.348
	30	404.3	512.7	1.268
	50	384.2	501.2	1.305
	59	370.3	490.2	1.324
	73	365.6	503.8	1.378
4	17	433.0	568.6	1.313
	23	403.5	519.2	1.287
	31	384.3	497.0	1.293
	37	445.8	551.4	1.237
	56	401.4	526.6	1.312
	66	400.8	502.4	1.253
	79	418.5	540.3	1.291
5	8	426.8	567.6	1.330
	33	412.0	511.7	1.242
	45	431.2	521.2	1.209
	46	432.7	558.4	1.291
	55	448.3	549.8	1.226
	68	471.4	596.6	1.266
	71	463.7	562.3	1.213

Appendix Table 12. Broiler chicken trial.

Diet	Pen	Body weight gain g/bird	Feed intake g/bird	Feed to gain ratio
6	5	444.1	560.6	1.262
	15	439.1	532.1	1.212
	22	476.4	612.0	1.284
	32	446.5	594.0	1.330
	39	405.6	524.6	1.293
	60	405.8	544.0	1.341
	63	444.1	574.8	1.294
	70	--	--	--
7	9	427.2	541.6	1.268
	19	458.7	558.0	1.216
	21	414.0	525.9	1.279
	29	410.6	491.3	1.197
	36	392.0	479.0	1.222
	49	469.1	567.4	1.210
	53	420.4	519.2	1.235
	62	465.3	558.5	1.200
8	2	451.2	558.4	1.238
	11	462.2	570.5	1.234
	20	408.3	536.0	1.313
	28	406.4	538.0	1.324
	48	397.3	527.8	1.328
	64	438.9	553.2	1.260
	75	428.8	552.2	1.288
9	3	439.0	549.4	1.251
	10	462.3	565.6	1.223
	13	437.3	560.5	1.282
	38	437.3	529.9	1.212
	44	418.4	525.1	1.255
	54	443.8	551.1	1.232
	76	447.7	552.5	1.234
10	24	423.5	525.7	1.241
	27	438.3	541.0	1.234
	35	407.5	523.0	1.283
	43	437.4	574.4	1.313
	52	458.0	558.8	1.220
	74	437.1	547.6	1.253
	77	463.1	580.5	1.254