THE EFFECTS OF PHENOBARBITAL, A DRUG USED TO TREAT EPILEPSY, ON INTESTINAL FLORA, BIFIDOBACTERIUM SP.

By

KAYLEEN R. TUCKER

Bachelor of Science

University of Nebraska

Lincoln, Nebraska

1998

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the degree of
MASTERS OF SCIENCE
December 2000

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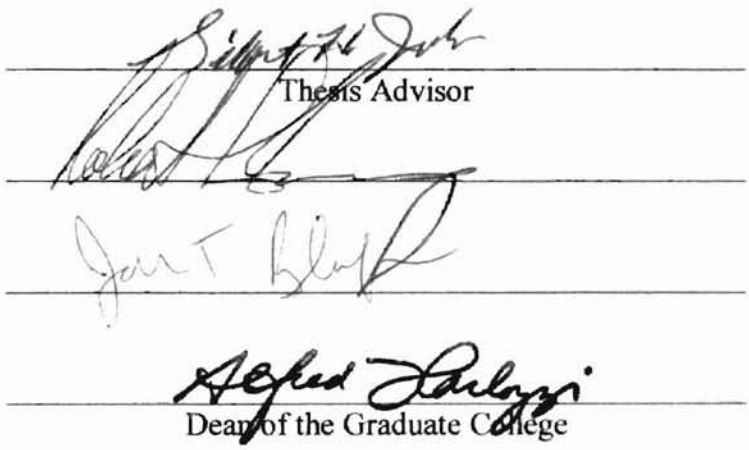
Thesis Approved:



Thesis Advisor

Dean of the Graduate College

ACKNOWLEDGEMENTS

I would like to acknowledge the multitude of people that have contributed to the contents of this thesis, physically and emotionally. There are many graduate students of the Entomology and Microbiology and Molecular Genetics departments who have helped throughout the writing of this thesis that I would like to acknowledge. Behind every student, is a driving force that keeps him or her motivated to study or on task to finish the writing of a thesis. For this, I would like to thank my parents, James and Connie Tucker who have always supported me in accomplishing the goals I have set for myself. I dedicate this thesis and degree to my parents. Last, but definitely not least, I would like to thank my fiancé, Charles Joyce for his love and support throughout this graduate school adventure ride.

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CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

Overview of Study

Scope and purpose. Collectively, microbes exhibit unparalleled metabolic diversity and adaptability, allowing them to survive in environments incompatible with large life forms. Microbes produce energy and carry out metabolism from a tremendous variety of organic materials and, in addition, can use radiant energy. Microbes can survive in the hot springs of Yellowstone Park and in Antarctica, in soil and in water, in the bottom of lakes and in compost heaps. and within and on the surfaces of both animals and plants. The ability of microbes to adapt and survive in a diversity of environments exceeds, by orders of magnitude, the survival ability of large life forms. Although, from the morphological viewpoint, microbes are simple, they are metabolically and physiologically complex. In contrast, collectively, large forms, although structurally complex, are, relatively speaking, physiologically simple. It is precisely because of the physiological and metabolic complexity of microbes that they can survive in such a wider variety of circumstances than can elephants and other large forms (6).

The metabolic capacity of the gut bacteria is extremely diverse (14, 36). Studies on the metabolic fate of drugs and foreign organic compounds up to the present have dealt almost exclusively with the changes occurring in the tissues. These reactions include oxidation, reduction, hydrolysis and conjugation and have been the subject of a review by GILLETTE (1963) (13, 34).

As complex as the twists and turns of the intestinal tract, so is the diversity of the microorganisms that inhabit it. (See Table 1) In man, the small intestine is sparsely

populated with microorganisms, most of which are aerobic or facultatively anaerobic in nature. Below the ileocecal valve, the proportion of anaerobic microorganisms increases to the point where they outnumber aerobes and facultative anaerobes by a factor of 10^3 to 10^4 (9, 36).

Any compound taken orally, any substance entering the intestine via the biliary tract or the bloodstream, or any substance secreted directly into the lumen is a potential substrate for bacterial transformation (14). Xenobiotics are foreign chemicals that are man made or of natural origin, such as drugs, pesticides, or environmental pollutants (21). The human body has a number of biochemical processes, located in the kidney, liver and gut that convert lipophilic compounds to more hydrophilic metabolites, and eliminate them from the body via urine, feces, or expired air. The key to understanding the role of intestinal microflora is to characterize the protein inductions by a xenobiotic, and their possible role in metabolism (7).

Introduction to the Two Studies

Screening of Intestinal Flora, *Bifidobacterium sp.*, for the Presence of a Drug Metabolizing Enzyme Complex

Drug metabolizing enzymes are responsible for the increased or decreased toxicity of foreign compounds, or xenobiotics. Xenobiotics are compounds that are not used as energy substrates or as building blocks for biological matrices(36). One enzyme that has been extensively studied in this manner is the Cytochrome P450 system. The cytochrome P450 enzyme family is responsible for the metabolism of a large number of drugs and environmental pollutants in mammals (35). Cytochrome P450, initially identified as a pigment from liver with a characteristic absorption peak at 450 nm when bound in the reduced state to carbon monoxide, is actually a multigene family of heme proteins that are found in essentially all eukaryotes and in some bacteria (12).

The cytochromes P-450 (P-450s) constitute an extremely large family ('superfamily') of haemoproteins that catalyze the oxidation of a wide range of physiological and non-physiological compounds(31, 41). All have characteristic ferrous carbon monoxide complex peaks near 450 nm, have monomeric molecular weights of about 50,000, and accept electrons from the flavoprotein NADPH-P-450 reductase(10, 18). A remarkable feature of the P-450's is the manipulation of the same basic structure and chemistry to achieve an enormous range of functions in organisms as diverse as bacteria and man. Indeed, the P-450s have been described as 'the most versatile biological catalyst known' (30). This is due to cytochrome P450s ability to bind with a variety of substrates and the conversion to new compounds. In addition to metabolizing several foreign compounds, the cytochrome P450 superfamily has a role in the

deactivation and activation of chemical carcinogens. The majority of toxic and carcinogenic chemicals do not produce their detrimental effects by themselves, except perhaps in small doses. In most cases, activation to electrophilic forms is necessary to produce molecules capable of reacting irreversibly with tissue nucleophiles (19). Considerable evidence has now been accumulated in support of the view that changes in P-450 composition can affect *in vitro* and *in vivo* metabolism of drugs in both animal models and humans(24, 32). The evidence is less clear in the case of carcinogens, which cannot be dealt with so easily in humans (18). During the past several years, it has become widely recognized that marked species differences occur among the foreign compound-metabolizing P-450s (15). Mammals are unique in that they possess their own eukaryotic P-450 system while some of the microorganisms that live symbiotically in animal gut, can possess their own bacterial P450 enzyme system(8).

Even though the microsomal cytochrome P-450 is classed as an oxygenase(11), it also catalyzes the *reductive biotransformation* of certain xenobiotics. These reactions proceed most readily under conditions of low oxygen tension. Owing to the transfer to reducing equivalents in cytochrome P-450-catalyzed reactions, certain xenobiotic substrates may accept one or two of these electrons. In effect, the substrate rather than molecular oxygen accepts the electrons and is reduced(23).

Intestinal microflora are also known to mediate the reduction of a number of chemicals, particularly those with azo or nitro groups(36). These microbes have virtually all the enzymatic machinery to mimic the cytochrome P-450-mediated reactions observed in mammalian systems(42). Owing to the anaerobic environment and the high concentration of chemical seen upon ingestion or biliary excretion, these microbes can

have a substantial effect on the *in vivo* biotransformation of xenobiotics. These microbes may also further modify metabolites of xenobiotics that were produced by hepatic cytochrome P-450. In some instances, a new reductive metabolite may be reabsorbed and further processed by hepatic enzymes (23).

A number of microorganisms possess monooxygenases similar to those found in mammalian tissues. Interestingly, the closest correlation exists between eukaryotic organisms and mammals (both possessing monooxygenases) in contrast to prokaryotic microorganisms which seem to produce a preponderance of dioxygenase enzymes (36).

After passage through the liver, drugs and other foreign compounds (or their metabolites) can pass into the systemic circulation where they may ultimately be eliminated by the kidneys. Alternatively, compounds may be excreted into the intestine via the bile. In some instances, reabsorption and enterohepatic circulation between the liver and intestine may occur (36). For the purpose of this paper, biliary secretion can be thought of as a mechanism whereby xenobiotics and their metabolites come in contact with intestinal microflora.

The Metabolism of Phenobarbital, a Drug Used for Epilepsy, by Intestinal Flora, *Bifidobacterium adolescentis* and *Bifidobacterium bifidum.*

Phenobarbital is an antiepileptic drug used to treat tonic clonic and partial seizures. As an effective stimulator of P450 enzymes, its use leads to increased metabolism of several drugs, including other antiepileptics, carbamazepine, and valproic acid(37, 38). The long elimination half-life of phenobarbital means that the drug must be administered in a loading dose to rapidly achieve a therapeutic blood concentration(40). The drug is addictive, and evidence of a withdrawal "syndrome" may present if the drug is discontinued suddenly. The most troublesome adverse effects of phenobarbital are sedation and negative effects on cognition, particularly in children (40).

The primary site of action for phenobarbital appears to be the motor cortex where the spread of seizure activity is inhibited. Possibly by promoting sodium efflux from neurons, it tends to stabilize the threshold, caused by K+, against hyperexcitability caused by excessive stimulation or environmental changes capable of reducing membrane sodium gradient. Phenobarbital reduces the maximal activity of the brain stem centers responsible for the tonic phase of tonic-clonic (grand mal) seizures(2).

The primary pathway for the metabolism of phenobarbital is hydroxylation of the phenyl ring. Butler (5) isolated *p*-hydroxy-phenobarbital in pure form from the urine of man and canine and proved its structure by synthesis. The extent to which phenobarbital is converted to this compound and its conjugates are not known. However, Butler noted that in urine specimens the concentration of the metabolite was more than five times higher than that of the drug. Based on this evidence, it is believed that the *para*-hydroxy derivative is the major metabolite of phenobarbital (43). Other research indicates that

phenobarbital contains two primary metabolites, *para*hydroxyphenobarbital and phenobarbital *N*-glucoside (1).

The significance of this study is that *Bifidobacterium* is a major intestinal strain commonly found in many individuals and its ability to metabolize xenobiotics, such as phenobarbital, may have a profound effect on the physiology of the host. Secondly, as probiotic therapy is becoming more popular, it is important that we understand the different metabolic systems that are found in wild strains (ATCC *Bifidobacterium*) and probiotic strains (*Bifidobacterium sp.*). Lastly, characterizing *Bifidobacterium's* metabolic capacity in terms of whether enzyme systems, such as cytochrome P450, exist is important as they may participate in the metabolism and generation of detoxified or toxified products, which can have a positive or negative effect on human health.

Table 1: Representation of the gastrointestinal microflora (33).

Microorganisms	Description
1. *Bacteroides*	Gram-negative, strictly anaerobic, non-spore forming rods
2. Lactobacilli 1. Anaerobic (*Bifidobacterium*) 2. Aerobic	Gram-positive rods
Fusobacterium	Gram-negative, strictly anaerobic, spindle shaped rods
Enterobacteria 1. *Escherichia coli* 2. *Aerobacter* 3. *Klebsiella* 4. *Proteus* 5. Providence group	Gram-negative, aerobic or facultatively anaerobic, non-spore forming rods
1. Clostridia	Gram-positive, anaerobic, spore-forming rods
2. Streptococci 1. Enterococci 2. Anaerobic	Gram-positive, aerobic or facultatively anaerobic cocci Gram-positive, anaerobic cocci
3. Pseudomonads *1. Pseudomonas* *2. Alcaligenes faecalis*	Gram-negative, aerobic, motile rods
4. Staphylococci	Gram-positive, aerobic or facultatively anaerobic cocci
5. Veillonella	Gram-negative, anaerobic cocci
6. Yeast	

CHAPTER 2

IDENTIFICATION OF PHENOBARBITAL-INDUCED PROTEINS FROM *BIFIDOBACTERIUM SPP.*

Introduction. The objective of this study is to determine if phenobarbital, an inducer of cytochrome P450, causes an induction of proteins. The strains were selected based on the results of quantitative and qualitative examination of the normal flora of 20 Japanese-Hawaiians(39). Eighteen of the most common strains studied by Moore were selected for our study. Five strains of *Bifidobacterium sp.* were tested. The chosen species were *B. adolescentis, B. bifidum, B. breve, B. infantis,* and *B. longum.* The following techniques were used to determine whether a protein induction, possibly cytochrome P450, was present. The techniques were anaerobic culture and growth, chemical induction, semi-pure protein extraction and determination, SDS-PAGE, silver stain, isoelectric focusing, and CO difference spectrum analysis.

Materials and Methods.

Inoculation and Growth of Anaerobic bacterial strains.

Five *Bifidobacterium spp.* were tested: *B. adolescentis, B. bifidum, B. breve, B. infantis,* and *B. longum.* A 1 ml amount of confluent *Bifidobacterium sp.* from chopped meat media was collected in a syringe and injected into a 15 ml test tube containing 7 ml of manufactured Anaerobe Systems Chopped Meat Carbohydrate with airtight Hungate Caps. The samples were incubated at 37°C for 48 hours, or until growth was evident by clouding of the test tube. Optical density was not taken. A 5 ml culture was then injected into an 18 ml KIMAX airtight test tube containing 5 ml of TYG media. This tube was

incubated at 37^0 Celsius for 48 hours. The TYG media was prepared using 5 g of tryptone, 2.5 g of yeast extract into 425 ml deionized water, 12.5 ml of salt solution 1, 12.5 ml of salt solution 2, 12.5 ml of $CaCl_2$, 2 ml of hemin, 1 ml of microminerals, and 5 ml of resazurin. The 500 ml solution was heated until boiling for 5 minutes in the microwave on a high setting, and degassed under N_2 for a minimum of 30 minutes. The TYG was cooled to approximately 40^oC, and 5ml of Vitamin K solution and 0.3g of Cysteine-HCl was mixed and added via syringe. The solution was adjusted to the pH of 7.0, and bubbled with N_2 for an additional 10 minutes. Simultaneously, to a 100ml bottle the following were added: 10ml Water, 10ml Volatile Fatty Acid Solution, and 0.2g Sodium Carbonate. The bottle was stoppered, sealed and autoclaved with the TYG media. Following sterilization, the TYG media was bubbled under N_2 until cooled to less than 50^0C. To the TYG, 20ml of 20% Glucose, 10ml of Reducing Agent (optional), and 1ml of 10x Vitamins was aseptically added. The sterile TYG media was placed into the N_2 37^oC incubator overnight. A 5ml amount of each *Bifidobacterium sp.* was added to each 500ml bottle of sterile TYG media. To verify purity, a gram stain and visual check for consistency was performed on the *Bifidobacterium* inoculum prior to inoculation of the TYG media.

Induction of *Bifidobacterium spp.* with Phenobarbital.

A 0.58g amount of Phenobarbital (5-Ethyl-5-phenyl-2,4,6-trioxohexahydropyrimidine) was dissolved in 10ml of anaerobic ddH_2O. Using a 0.20 μM filter and syringe, 1 ml of the phenobarbital solution was injected into the 500 ml TYG container making a final 5

mM phenobarbital concentration. All additions were performed inside the anaerobic nitrogen chamber. The sample was incubated for 24-48 hours. To ensure purity, a gram stain was performed, following incubation.

Acquisition of Supernatants I and II.

Because *Bifidobacterium spp.* are anaerobic bacteria, all procedures were performed anaerobically. All dishware (bottles, pipettes, pipette tips, etc) were placed into the anaerobic nitrogen chamber at least 24 hours prior to use, which allowed them to adapt/adjust to the anaerobic conditions. A 1 L amount of MOPS Anaerobic Buffer was prepared using 100 mM MOPS, 10% glycerol, 1 mM EDTA. The solution was adjusted to a pH of 7.3 and heated to boiling in the microwave for 6 min/500 ml. The solution was degassed under N_2 for 60 min. Following sterilization, the solution was degassed under N_2 for 5 h. Prior to storage in the anaerobic chamber, a 0.2 mM amount of the reductant, DTT, dissolved in 2 ml of anaerobic water and added to the solution.

After sufficient growth was present in the TYG culture bottles, the cell culture was poured into two 250ml Nalgene bottles. The cells were pelleted by spinning at 10,000 rpm for 15 min at 4°C with a JA-20 Beckman rotor. The pellet was resuspended in MOPS buffer (10ml MOPS/250 ml culture pellet). The suspension was transferred to a 50ml centrifuge tube and capped.

The cells were pelleted by spinning at 10,000 rpm for 15 min at 4°C. The pellet was again resuspended in MOPS buffer (10ml/250ml culture). Note: The volume can be adjusted based on the pellet size. The supernatant was removed and 10ml MOPS buffer was added. A 10 µl amount of mutanolysin was added and the pellet plus buffer solution

was incubated at 37°C for 2h. Next, the samples were placed on ice to cool for approximately 2 min. The samples were sonicated, using a flat tip sonicator probe. The sonicator was set at 2 sec pulses, 60% output control and pulsed 15-25X with a flat tip. To avoid excessive foaming and heat, the sample was mixed gently and placed on ice for 1 min following each round of pulsing. Sonication was repeated 2 to 4 more times. The sonicated sample was then poured into an ultracentrifuge tube. The sample was spun at 31,000 rpm at 4°C for 35 min in a Ti50.2 Beckman rotor. The pellet was resuspended in 10ml MOPS (250ml culture) and transferred into a plastic ultracentrifuge tube. The pellet was broken up by homogenization. A 0.05g amount (0.5% final conc.) of CHAPS was added directly to each 10ml sample and stirred at 4°C overnight. The following day, the sample was spun at 31,000 rpm for 35 min at 4°C. The supernatant was collected to perform a reduced CO Spectrum on each sample. The supernatants were stored at –80°C.

Determination of Protein Concentrations of Supernatants using the Folin Reaction. Reagent A contained 2g of Na_2CO_3 in 100 ml of 0.1N NaOH. Reagent B contained 0.05g of $CuSO_4$-$5H_20$ in 10 ml of 1% (W/V) solution of Sodium Tartrate. Reagent C consisted of a combination of 50ml of Reagent A mixed with 1ml of Reagent B. Reagent D was comprised of 1 N Folin-Phenol. A 100 µl amount of the protein sample was added to 300 µl of water. Next, 2ml of Reagent C was added and the solution was vortexed. The sample was left to stand for 10 min. A 200 µl amount of Reagent D was added, vortexed and left to stand for 30 min. The samples were read at 750 nm with a Shimadzu UV-1601PC (Columbia, MD). In this experiment, bovine serum albumin (BSA) was used as the control.

Protein Preparation for SDS-PAGE.

The calculations were paired up with the correct corresponding protein sample. If the sample was concentrated (> 150 μg), a buffer consisting of deionized water was added to make the final volume equal 150μg/μl. If the sample was too dilute (< 150μg), it was split into 150 μl aliquots and two or more simultaneous preparations were carried out.

Each 150 μl sample was placed into a 1.5 ml microcentrifuge tube and the following was added: 600 μl methanol, 150 μl chloroform, and 450 μl water. The sample was vortexed for 1 minute then spun at 14,000 rpm for 2 minutes. The upper layer was discarded, leaving only the interface and lower layer. If samples were originally too dilute, the multiple aliquots were combined into one sample tube. To the interface and lower layer, 300 μl of methanol was added and the tube was inverted four times to help clear the sides of the microcentrifuge tubes of any protein debris. The sample was spun at 14000 rpm for 3 mins. The supernatant was removed and the pellet was dried in a speed vacuum with a low heat setting for 10 mins to remove the residual methanol. To the dry pellet, 20 μl of water and 5 μl of tracking dye was added. The sample was vortexed briefly, boiled for 5 min, vortexed again then spun for 1 minute to pellet insoluble debris. The sample was then ready for electrophoresis.

SDS-PAGE procedure.

The running and stacking gels were prepared (40% acrylamide, 0.5 and 1.5 mM Tris buffer, water, 10% SDS, 10% APS, and TEMED) and loaded with the protein samples, along with a BIORAD SDS-PAGE molecular weight standard, broad range. The samples

were run at 200 V for 45 min. Following electrophoresis, the gels were placed into a Coomassie Blue Staining solution for 24 hrs. The following day, the gels were destained with a 50/50 water-methanol solution. The final product was vacuum dried for data evaluation and analysis.

Silver Stain Procedure.

The gels were silver stained using the manufactured BIORAD Silver Stain Plus Kit(16). This procedure included four main steps: Fixative Step, Rinse Step, Staining and Developing Step, and the Stop Step. The reagents were provided and steps were followed exactly to the corresponding manual(16). The SDS-PAGE gels, stained with coomassie blue, were destained prior to the start of the silver staining procedure.

Rotofor Cell Procedure.

The BIORAD Rotofor cell is an isoelectric focusing apparatus used for protein separation and purification. This apparatus incorporates a cylindrical focusing chamber with an internal ceramic cooling finger. Anion and Cation Exchange solutions were degassed for 10 min prior to the assembly of apparatus. The apparatus was equilibrated using a sample run containing deionized water. The BIORAD Rotofor Cell Instruction Manual was followed completely for the loading, running, and harvesting of the protein samples. The 150 μg protein sample, which contained ampholytes and urea were loaded into the apparatus, using a large syringe. Any air bubbles that collected while loading the chamber, were removed prior to the running of the procedure. The power supply was set at 12 W for approximately 4 hours. The samples were harvested by a vacuum source into

fraction tubes. The pH was tested and recorded for each sample prior to labeling and storage.

Results.

Based on the following procedures, SDS-PAGE gels, CO Spectrums, and Silver Stains, the following results for each strain are provided. For each strain, the four supernatants were examined. The four supernatants are: N1 (non-treated, supernatant 1), I1 (phenobarbital-induced, supernatant 1), N2 (non-treated, supernatant 2), and I2 (phenobarbital-induced, supernatant 2). Supernatant 1 represents the cytoplasmic proteins whereas supernatant 2 consists of the membrane-bound proteins.

Bifidobacterium adolescentis. The protein concentrations were 141 μg for N1, 144 μg for I1, 315 μg for N2, and 223 μg for I2. *B. adolescentis* showed no protein induction based on the 10% SDS-PAGE gel, stained with Coomassie blue (Fig. 1). There were also no inductions present on the Silver Stain gel (data not shown). No peaks, indicative of the presence of a cytochrome, were found with the CO Spectrum assay (data not shown).

Bifidobacterium bifidum. A protein induction was observed in the induced, supernatant 2 lane(I2), at 43 kDa. On the Silver Stain gel, two proteins were identified at 45 and 65 kDa (data not shown). For supernatants I1, N2, and I2, a peak around 416-420 nm was seen. However, the absorbancy values were low at -0.028, 0.005, and –0.046 respectively (data not shown). Previous findings by other researchers, showed an

induction in supernatant I1 at 40 and 48 kDa. For supernatant I2, previous gels show an induction at ~33 kDa (data not shown).

Bifidobacterium breve. No protein inductions were seen for *B. breve* on the Silver Stain (data not shown) or SDS-PAGE gels (Fig. 3 &4). For supernatant I2, a peak at 417 nm was identified with an absorbance of 0.020 (data not shown). Previous SDS-PAGE gels showed an induction in supernatant I1 at 40 kDa and in I2 at 38 kDa.

Bifidobacterium infantis. For *B. infantis*, an induction was seen at approximately 180 kDa for supernatant I2 (Fig. 3). There was no detection of an induction in any of the Silver Stain gels (data not shown). A CO Spectrum peak was observed at approximately 416 nm for supernatant N1 with an absorbancy of 0.02. For supernatant N2, a peak was observed at 430 nm with an absorbance of 0.05 (data not shown). Previous work showed SDS-PAGE gel protein inductions in supernatant I1 at 35 kDa. This sample was evaluated further, using better separation techniques, such as the Rotofor, followed by a Silver Stain. However, after isoelectric focussing with the Rotofor, evidence of the previous induction at 35 kDa was absent. The silver stain gel confirmed that the suspected induced protein was unable to be isolated and identified. This could be due to the sample being lost during loading or harvesting of the Rotofor chamber.

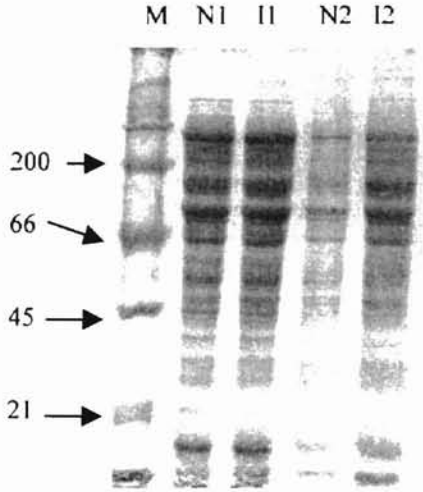
Bifidobacterium longum. No inductions were observed for SDS-PAGE (Fig. 4) and Silver Stain gels, or CO Spectrum profiles (data not shown). It should be noted that on the SDS-PAGE gels, supernatants N1 and I1, the tracking dye was too dilute to detect

any reliable results. Therefore, the SDS-PAGE gel was repeated, still consistent with previous negative results.

Conclusions.

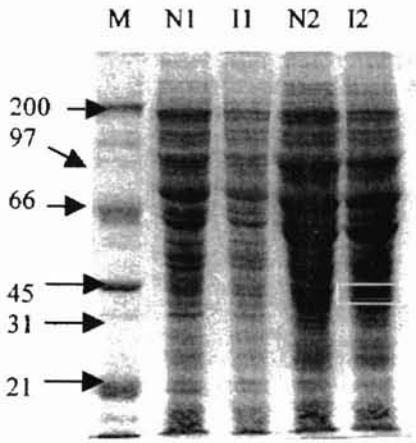
Of the five *Bifidobacterium spp.* that were tested, no significant protein inductions were observed. A significant finding of a protein induction is one that shows up consistently in the SDS-PAGE and Silver Stain gels for the same supernatant and molecular weight. This would eliminate the questionability as to whether the protein induction was present or not. Also, a true induction should be absent in the untreated supernatants. These findings indicate that there are no significant inductions in any of the five strains tested. Of the protein inductions that were seen, none have shown to be repeatable or present in both gel procedures. A CO spectrum peak around 416 to 425 nm is indicative of a cytochrome presence. However, the absorbance value must be significant in order to suggest an induction by a cytochrome P-450. Of the CO spectrum peaks observed in the 416-425 nm range, none have shown an indication of the presence of a cytochrome P-450 protein. Of the *Bifidobacterium spp.* that were tested, our results show that phenobarbital had no significant effect on the induction of a cytochrome P450 protein. Evidence of a cytochrome P450 complex in our samples was not detected. Because of the lack of evidence for a cytochrome P450 present in our samples, we considered the alternative hypothesis that another complex, or reaction is responsible for the degradation of phenobarbital. This could be another unidentified system analogous to the inducible lac operon system that allows the metabolism of lactose in *E. coli*(4).

Figure 1. SDS-PAGE gel of *B. adolescentis*



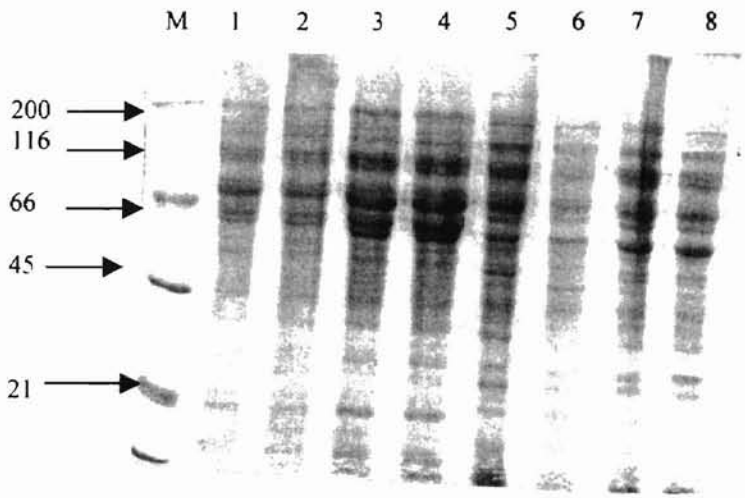
10% SDS-PAGE gel of *B. adolescentis* protein fractions. M is molecular weight marker (in kDa.), N1 is non-treated, fraction 1, I1 is phenobarbital-treated, fraction 1, N2 is non-treated, fraction 2 and I2 is phenobarbital-treated, fraction 2.

Figure 2. SDS-PAGE gel of *B. bifidum*



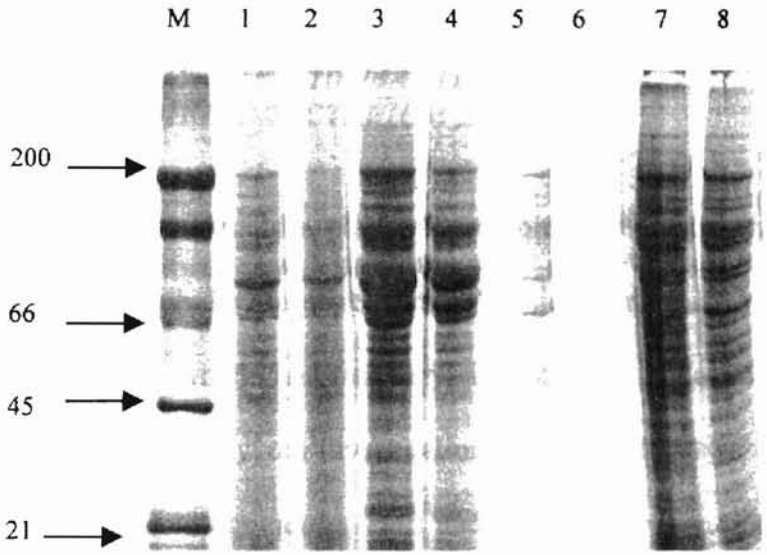
10% SDS-PAGE gel of *B. bifidum* protein fractions. M is the molecular weight marker (in kDa.), N1 is non-treated, fraction 1, I1 is phenobarbital-treated, fraction 1, N2 is non-treated, fraction 2, and I2 is phenobarbital-treated, fraction 2. A protein induction is indicated by a ▭

Figure 3. SDS-PAGE gel of *B. breve and B. infantis*



M is the molecular weight marker (in kDa), Lanes 1 through 4 are *B. breve* and Lanes 5 through 8 are *B. infantis*. Lanes 1 and 5 are non-treated, fraction 1, lanes 2 and 6 are phenobarbital-treated, fraction 1, lanes 3 and 7 are non-treated, fraction 2, and lanes 4 and 8 are phenobarbital-treated, fraction 2. A protein induction is indicated by a ▭

Figure 4. SDS-PAGE gel of *B. breve and B. longum*



M is the molecular weight marker (in kDa.). Lanes 1 through 4 are *B. breve* and lanes 5 through 8 are *B. longum*. Lanes 1 and 5 are non-treated, fraction 1, lanes 2 and 6 are phenobarbital-treated, fraction 1, lanes 3 and 7 are non-treated, fraction 2, and lanes 4 and 8 are phenobarbital-treated fraction 2.

CHAPTER 3

THE METABOLISM OF PHENOBARBITAL, A DRUG USED FOR EPILEPSY, BY INTESTINAL FLORA, *BIFIDOBACTERIUM ADOLESCENTIS* AND *BIFIDOBACTERIUM BIFIDUM.*

Introduction. Human intestinal flora play a large role in metabolizing a variety of compounds that travel through the digestive tract(22). This contributes to their extremely diverse metabolic capacity, which unfortunately, is not fully understood. To better understand their metabolic capacity, our investigation has focused on two major intestinal microfloral strains, *Bifidobacterium adolescentis* and *Bifidobacterium bifidum*(39). Our hypothesis is based on previous reports that *Bifidobacterium* may possess a P450-like protein that may participate in the metabolism of various xenobiotics. The results from this study have determined the ability of these microbes, under anaerobic conditions, to metabolize Phenobarbital, a pharmaceutical drug used to treat epilepsy and a potent inducer of hepatic cytochrome P-450. The effects of a range of phenobarbital concentrations on bacterial growth were quantitated and the generated metabolite (benzeneacetamide) was analyzed by the GC/MS method. The results of this study demonstrated that phenobarbital inhibited the growth of the slower growing *B. bifidum* compared to the faster growing *B. adolescentis*. Both strains were able to metabolize phenobarbital to benzeneacetamide, but the faster growing *B. adolescentis* exhibited greater metabolizing activity. The results of this study demonstrate that intestinal flora can metabolize pharmaceuticals, which has significant clinical implications.

The metabolic capacity of intestinal microorganisms is vast and not well understood (14, 28). The intestine contains a massive, as well as, a variety of bacterial genera, which participate in the metabolism of different xenobiotics. *Bifidobacterium*

species are considered a major constituent in the small and large intestine. They are gram-positive bacteria, irregular rod shape, and strict anaerobes, which allow for their predominance as normal gut flora near the villi of the colon (33). In addition, they are known to have a positive effect on the host, as they help maintain the intestinal microbial balance, as well as discourage other pathogens from colonizing.

Each day, our bodies take up significant amounts of material that are not used as energy substrates or as building blocks for biological matrices. Xenobiotics are compounds that fall into this category. If these xenobiotics accumulated in an organism, the resulting accumulation could affect the physiological systems. Elimination of these products occurs by a process called biotransformation, in which phase I and phase II enzymes are involved. The major elimination pathway of these products involves excretion via bile, expired air, feces and urine.

The metabolism of phenobarbital occurs in the liver as a result of the CYP 2C cytochrome P450(17,25). The product generated is either the *para*-hydroxyphenobarbital (43) and/or phenobarbital *N*-glucoside (1). The extent to which phenobarbital is converted to this compound and its conjugates is not known (43). The purpose of this study is to determine if major intestinal strains, such as *Bifidobacterium,* are involved in the metabolism of phenobarbital.

The significance of this study is that *Bifidobacterium* is a major intestinal strain commonly found in many individuals and its ability to metabolize xenobiotics, such as phenobarbital, may have a profound effect on the physiology of the host. Secondly, as probiotic therapy is becoming more popular, it is important that we understand the different metabolic systems that are found in wild strains (ATCC *Bifidobacterium*) and

probiotic strains (*Bifidobacterium sp*). Lastly, characterizing *Bifidobacterium's* metabolic capacity in terms of whether enzymes systems such as cytochrome P450 exist is important as they may participate in the metabolism and generation of detoxified or toxified products, which can have a positive or negative affect on human health.

Materials and Methods.

Bacterial strains.

Bifidobacterium adolescentis ATCC 15703, *Bifidobacterium bifidum* ATCC 15696

Culture media and growth curves.

The reference strains were cultured in anaerobic Chopped Meat media for 48 hours. The cultures were then aseptically transferred to anaerobic TYG media and incubated for 24 hours at 37°C. Following incubation, 100 µl of cells were subcultured to 5 ml TYG anaerobic tubes containing different concentrations of phenobarbital (final concentrations 0 mM, 5 mM, 10 mM, and 20 mM). A gram stain was performed on each sample to ensure purity of the culture. A uniform group of gram-positive cells was considered an axenic culture. The experiments were performed in triplicate for all strains tested. The growth was determined by measuring the optical density (O.D.) at 600 nm using a Spec-20 (Spectronic 20-D). The Spec-20 was calibrated to zero absorbance by using blank media tubes with the appropriate concentration of phenobarbital (0 mM, 5 mM, 10 mM, and 20 mM). Time points were recorded between 0 to 100 hours. Following the growth curve, the samples were stored at -80°C. The O.D. data was compiled and graphed using

Microsoft Excel Chart Wizard. The protein profiles for each sample were analyzed by SDS-PAGE using a methanol-chloroform extraction procedure(21).

Gas Chromatography/ Mass Spectrometry.

The -80°C stored samples were also analyzed using GC/MS (Gas Chromatography/Mass Spectroscopy) (27). To a borosilicate culture tube containing 500 µl of sample, a 125µl amount of 1M K_2HPO_4 was added, followed by 3.25 ml of ether/chloroform (2:1) solution. This solution was vortexed for one minute and then centrifuged at 1200 rpm for 2 minutes. The organic phase (top layer) was retained and subjected to N_2 gas until dryness occurred. The sample was then reconstituted with 100 µl of ethyl acetate and appropriate dilutions were made. The samples were then injected into the GC/MS (HP5890, HP5989B) using a DB5, 30 meter column at 0.25 microns. The data was analyzed by using a digital integrator.

Results

Growth curves analysis.

Table 2 shows the doubling times for *Bifidobacterium adolescentis* (Fig. 5) and *Bifidobacterium bifidum* (Fig. 6) under different concentrations of phenobarbital. There was an overall increase in doubling time as the concentration of phenobarbital was increased. There was also an extended lag time for the 20 mM concentration of phenobarbital, while the lesser concentrations displayed a lag phase that resembled the control. The slope of the log phase for both *B. adolescentis* and *B. bifidum* were

calculated to determine if there was any change in the rate of growth for the various concentrations of phenobarbital (Fig. 7). The protein profiles for all samples showed no major induction of proteins (data not shown).

Gas Chromatography and Mass Spectrophotometry (GC/MS).

The -80°C samples were thawed and prepared for the GC/MS analysis. The GC data for both strains showed two major peaks at thirteen and eighteen and half minutes for all concentrations tested (Fig. 10,11,12,14,15,16). The 0mM Phenobarbital blank (Fig. 8 & 13), 5 mM phenobarbital degradation control (Fig. 9) and sterile media showed no major peaks. The MS identified the two major peaks as phenobarbital and benzeneacetamide. The phenobarbital has undergone reductive cleavage as the terminal nitrogen obtained a hydrogen atom. It appears the phenobarbital was cleaved at three positions, releasing the CH_3CH_2, and CONCO molecules (Fig. 19). The percent of product generated in relation to the parent compound was determined by measuring the area under the peaks and its presence over a period of time (Table 3). The absence of the benzeneacteamide peak in our control samples suggests the metabolism of phenobarbital is due to *Bifidobacterium*. For *B. adolescentis* (Fig. 18), about 40% of the phenobarbital was metabolized to benzeneacetamide in the 5 mM sample, 30% in the 10 mM sample, and 45% in the 20 mM sample. For *B. bifidum* about 10% of the phenobarbital was metabolized to benzeneacetamide in the 5 mM sample, 40% in the 10 mM sample, and 10% in the 20 mM sample. The ratio of metabolite, benzeneacetamide, to parent compound, phenobarbital, was also calculated to determine if metabolism of phenobarbital was fixed or variable dependent upon the concentration (Fig. 17). We found for *B. adolescentis*

that as the concentration of phenobarbital was increased, so did the yield of metabolite. However, for *B. bifidum*, the abundance of metabolite increased as concentration increased, except for 20 mM, in which the amount of metabolite was decreased.

Discussion.

Intestinal flora possess a diverse metabolic system due to their constant exposure to different xenobiotics(33, 34). Their metabolic products are either eliminated by way of feces or they are absorbed into the gastrointestinal mucosa and eventually make their way into the liver. The effects that intestinal flora have on the expression of liver enzymes such as cytochrome P450 has been documented(17). Altering the expression of cytochrome P450 can affect the detoxification and metabolite activation processes, which can impact human health. Therefore, our understanding of how specific intestinal microbes metabolize xenobiotics is important.

The objective of our study was to determine if phenobarbital, a potent inducer of cytochrome P450 and a drug used to treat epilepsy, was metabolized by *Bifidobacterium*, which is a major intestinal flora. Our results demonstrated an effect on the growth rate shown by an increase in the lag phase with increasing concentrations of phenobarbital. An extended lag phase was observed only for the most concentrated sample (20 mM), while the other samples (5mM, 10 mM) were slightly higher then the 0 mM sample. The growth rates of *B. bifidum* (22.38/hr) were significantly slower than *B. adolescentis* (7.17/hr). As a result, the percentage of product generated for the slower growing *B. bifidum* was overall less when compared to the faster growing *B. adolescentis*. In addition, phenobarbital seemed to affect the growth of the slower growing *B. bifidum*

more than the faster growing *B. adolescentis*. These results suggest the faster growing and more resistant *B. adolescentis* was able to metabolize phenobarbital more extensively than the slower growing and more sensitive *B. bifidum*. Overall, phenobarbital was metabolized by both strains.

The experimental concentrations of phenobarbital used in this study have a clinical significance. A 5 mM final concentration is comparable to the upper level dose of an average adult. Alternatively, the 20 mM concentration is analogous to that of a lethal dose to the average human. Therefore, the possibility of *Bifidobacterium* in the human intestinal tract metabolizing phenobarbital is likely. Also under in vivo conditions, the fate of benzeneacetamide is unknown. Because it contains a toxic benzene ring, the absorption and metabolism by liver gastrointestinal and/or liver enzymes is possible thereby releasing the toxic benzene molecule into the surrounding environment.

The unique finding of the study showed for the first time how *Bifidobacterium*, a major intestinal microflora, was capable of metabolizing phenobarbital into benzeneacetamide which is dramatically different then what occurs in mammals (Fig. 19) as phenobarbital is normally metabolized by the hepatic cytochrome P450 into *p*-hydroxy-phenobarbital and/or phenobarbital *N*-glucoside (1).

Overall, our results have clinical significance regarding the role of intestinal flora in the metabolism of foreign compounds, such as phenobarbital. Our results demonstrate that two strains. *Bifidobacterium adolescentis* and *B. bifidum*, metabolize phenobarbital to a potentially toxic substance, benzeneacetamide. Therefore, future studies should include more pharmaceutical testing involving intestinal flora, prior to drug approval.

Since *Bifidobacterium spp.* is a major component of the intestinal flora population that is able to metabolize phenobarbital to the compound, benzeneacetamide other clinical significance needs to be evaluated. There is no literature stating whether benzeneacetamide is an active metabolite of phenobarbital, indicating that the metabolism of phenobarbital could be dangerous to a patient that relies on the ability of phenobarbital to control seizures. Also, we have shown that an intestinal bacterium can metabolize the phenobarbital. Therefore, it leaves us to ask the question, what other compounds can be metabolized by intestinal flora?

Table 2

Growth characteristics

PB (mM)	Doubling time	
	B. adolescentis	*B. bifidum*
0	7.17	22.38
5	9.08	21.55
10	11.36	47.10
20	13.28	71.90

These values represent the period of time in which the microorganisms, *B. adolescentis* and *B. bifidum*, have a two-fold increase in the absorbance value. The change in time was measured by the $end_{log\ phase}$ minus $beginning_{log\ phase}$.

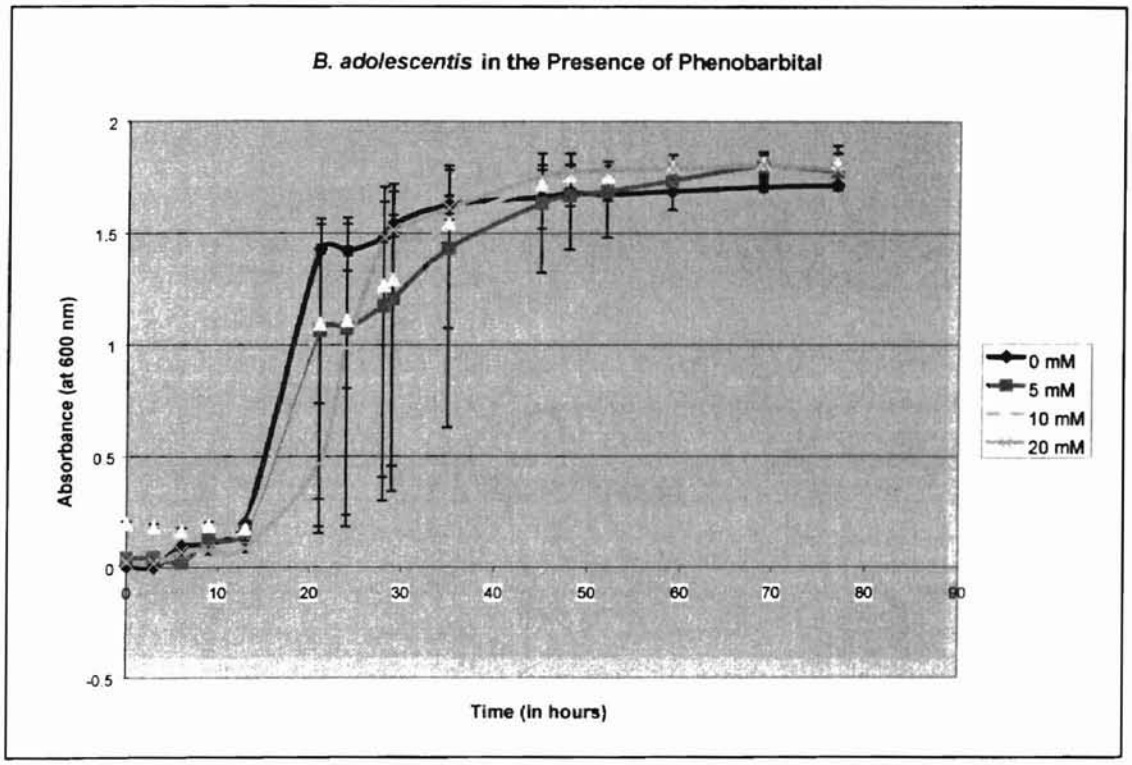
Table 3

Percentage of product generated

PB (mM)	Percent *B. adolescentis*	*B. bifidum*
0	0	0
5	40	10
10	30	40
20	45	10

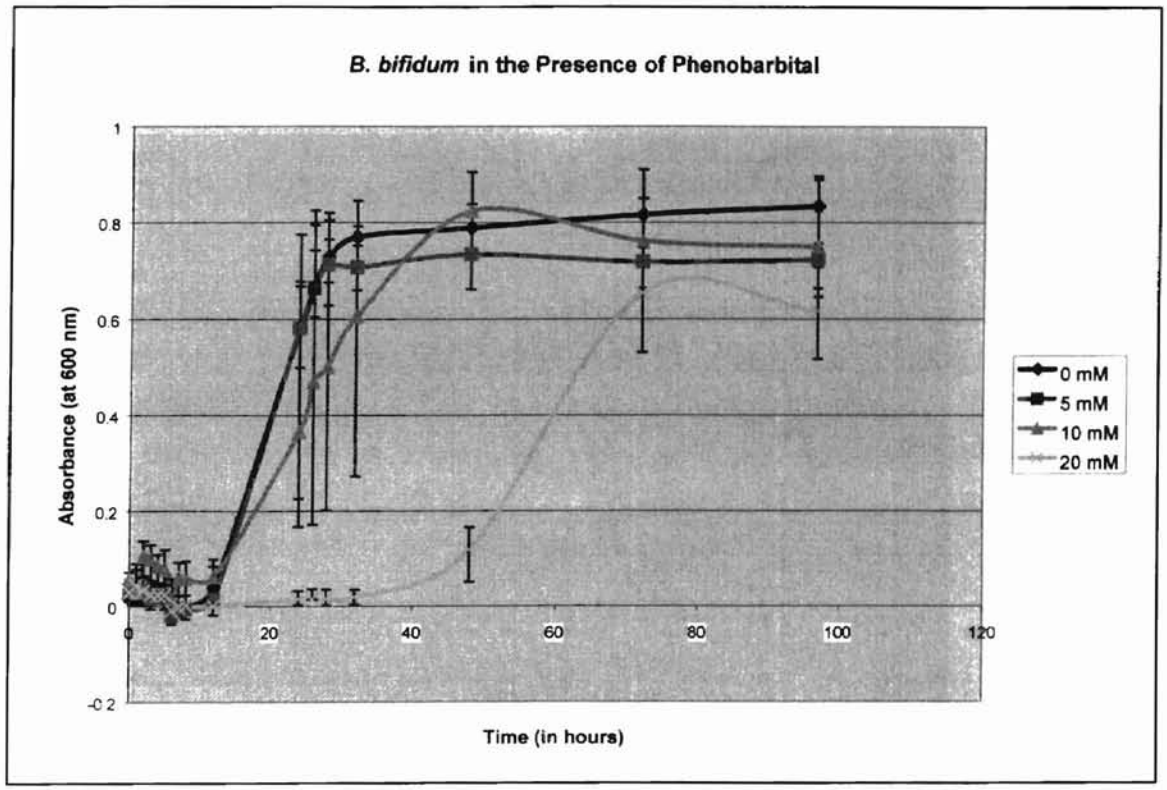
This table shows the percent of product, benzeneacetamide, that was produced when compared to the parent compound, phenobarbital.

Figure 5. Growth Curve of *B. adolescentis* in the Presence of Phenobarbital



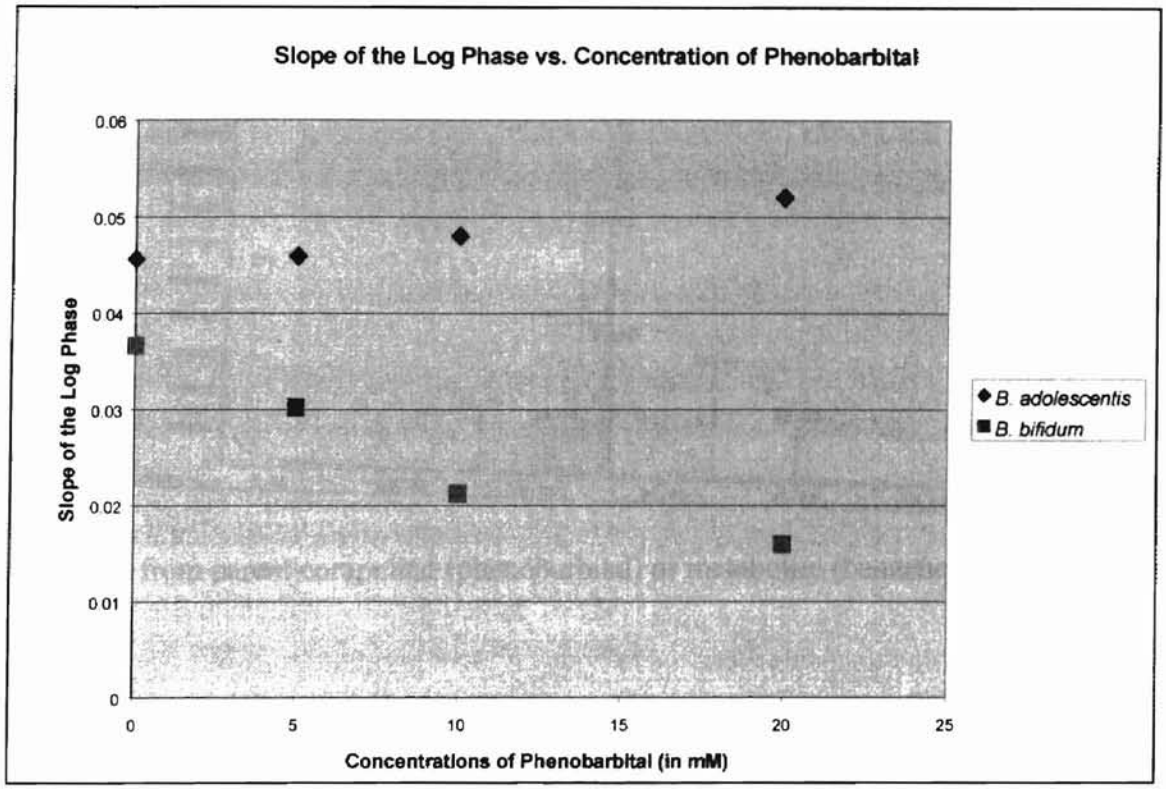
The y axis represents the absorbance at 600 nm and the x axis represents time, in hours. Four concentrations of phenobarbital were tested: 0 mM, 5 mM, 10 mM and 20 mM.

Figure 6. Growth Curve of *B. bifidum* in the Presence of Phenobarbital



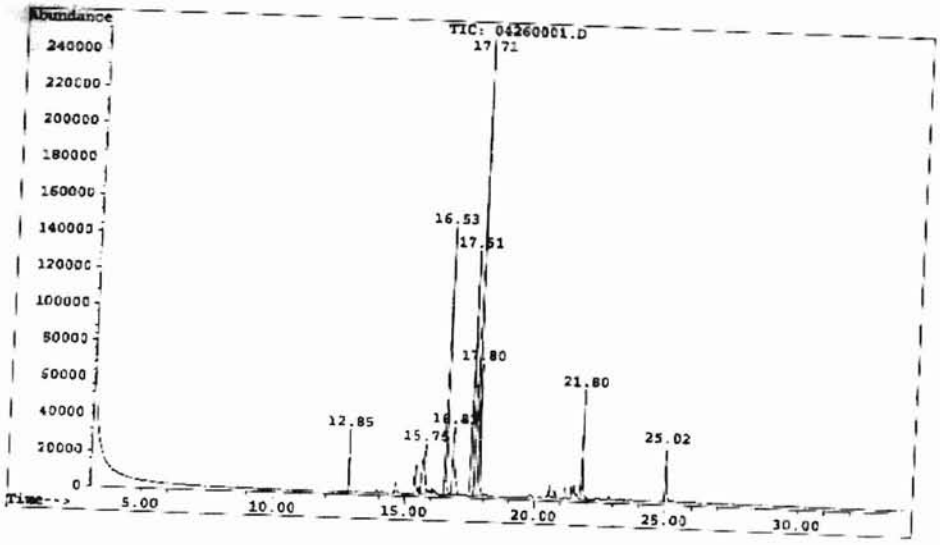
The y axis indicates absorbance at 600 nm, and the x axis is time, in hours. Four concentrations of phenobarbital were tested: 0 mM, 5 mM, 10 mM and 20 mM.

Figure 7. Slope of the Log Phase versus Various Concentrations of Phenobarbital



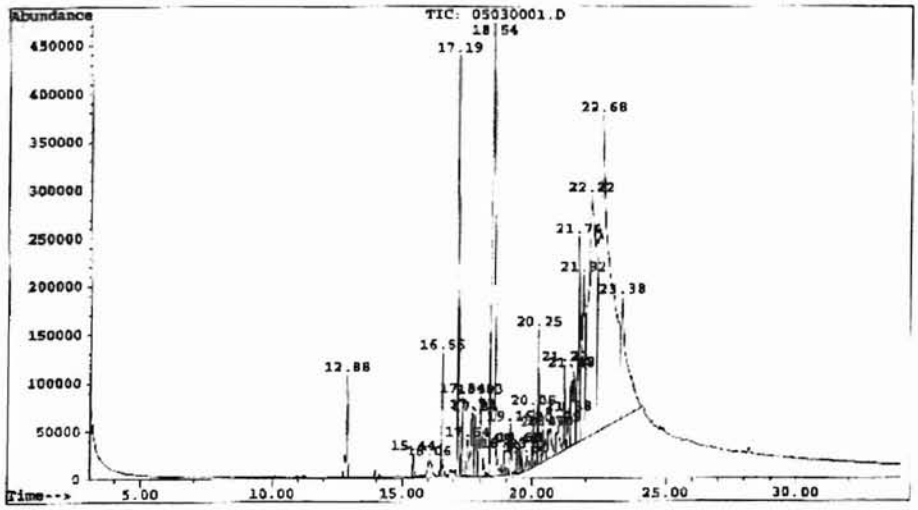
The slope of the log phase was calculated by taking the difference in the y axis divided by the x axis for only the log phase. For example, the absorbance at the start of the log phase was subtracted from the absorbance at the end of the log phase. This difference was divided by the change in time over the two points. The diamond shape represents *B. adolescentis* and the square is *B. bifidum*.

Figure 8. GC/MS Results for *B. adolescentis* 0 mM Phenobarbital



No peaks from parent compound (phenobarbital) or metabolite (benzeneacetamide) were detected.

Figure 9. GC/MS Results for *B. adolescentis* 5 mM Phenobarbital Degradation Control



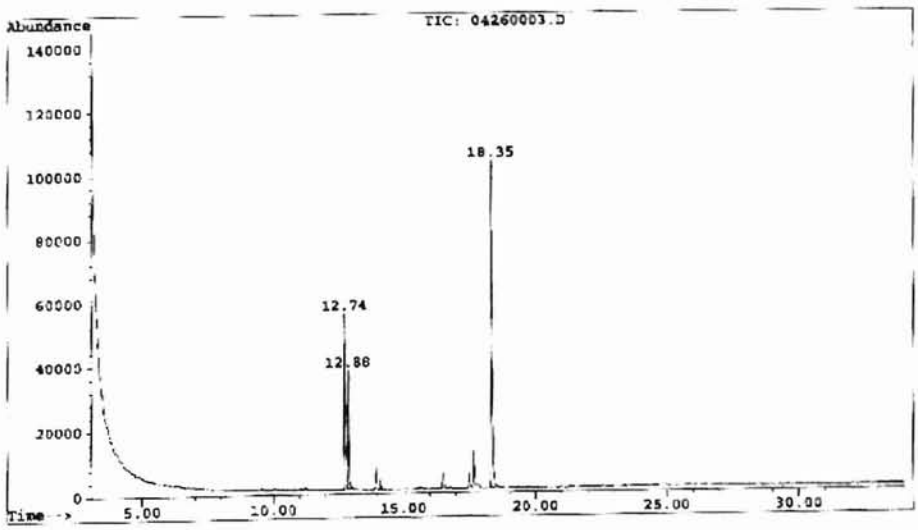
Major Peak Identification (in minutes)

18.54 represents phenobarbital

17.19 represents N-methylamino indole

12.88 represents butylated hydroxytoluene

Figure 10. GC/MS Results for *B. adolescentis* 5 mM Phenobarbital



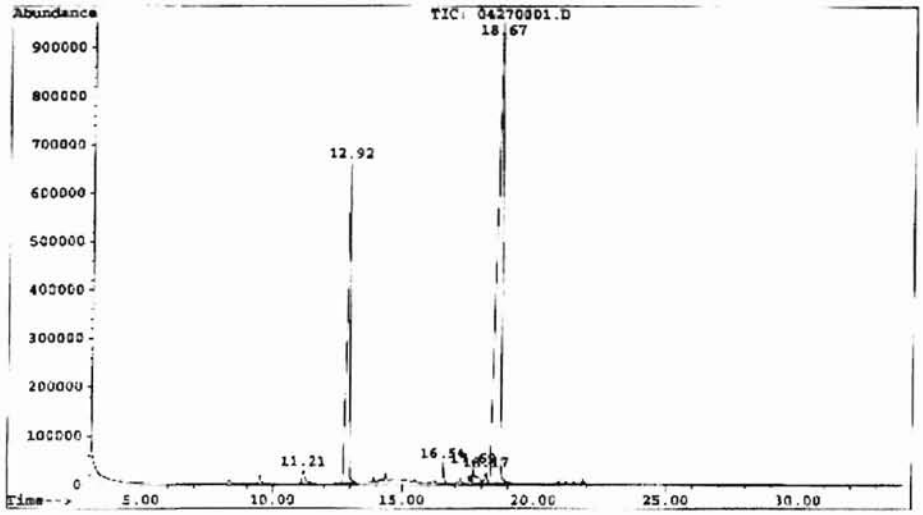
Major Peak Identification (in minutes)

18.35 represents phenobarbital

12.88 represents phenol

12.74 represents benzeneacetamide

Figure 11. GC/MS Results for *B. adolescentis* 10 mM Phenobarbital

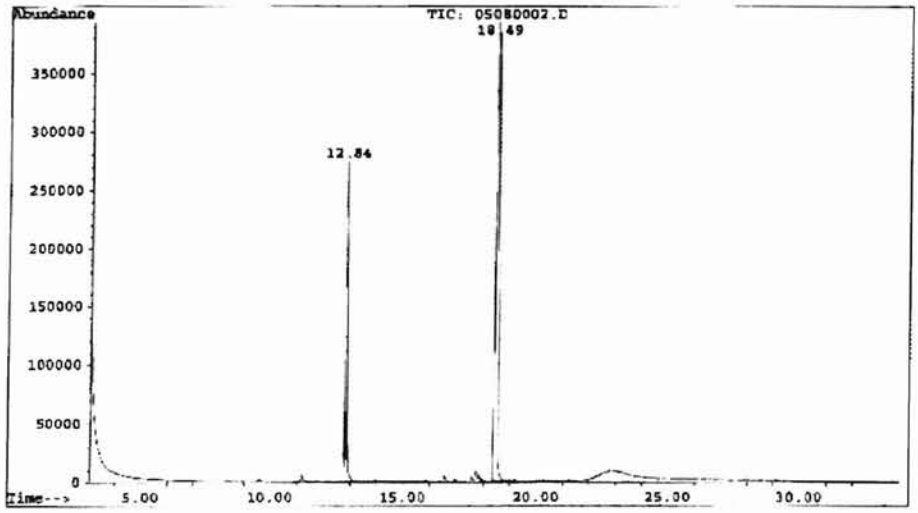


Major Peak Identification (in minutes)

18.67 represents phenobarbital

12.92 represents benzeneacetamide

Figure 12. GC/MS Results for *B. adolescentis* 20 mM Phenobarbital

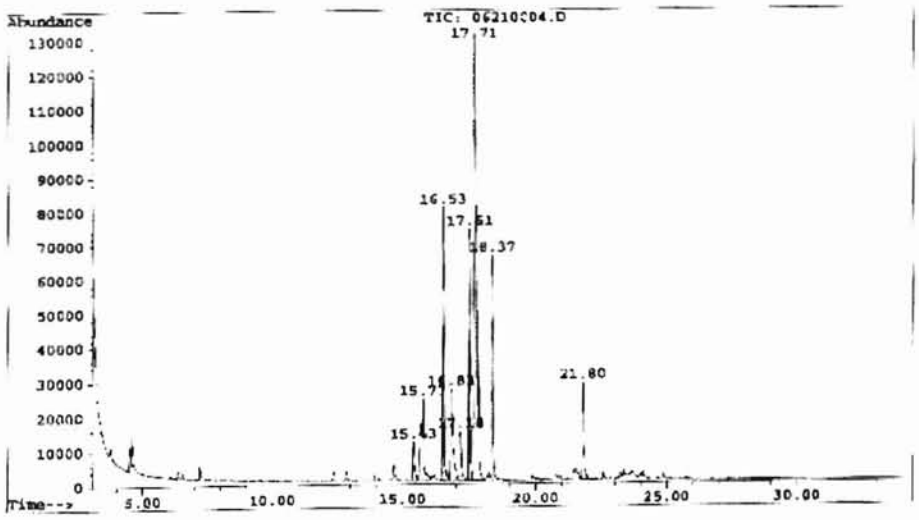


Major Peak Identification (in minutes)

18.49 represents phenobarbital

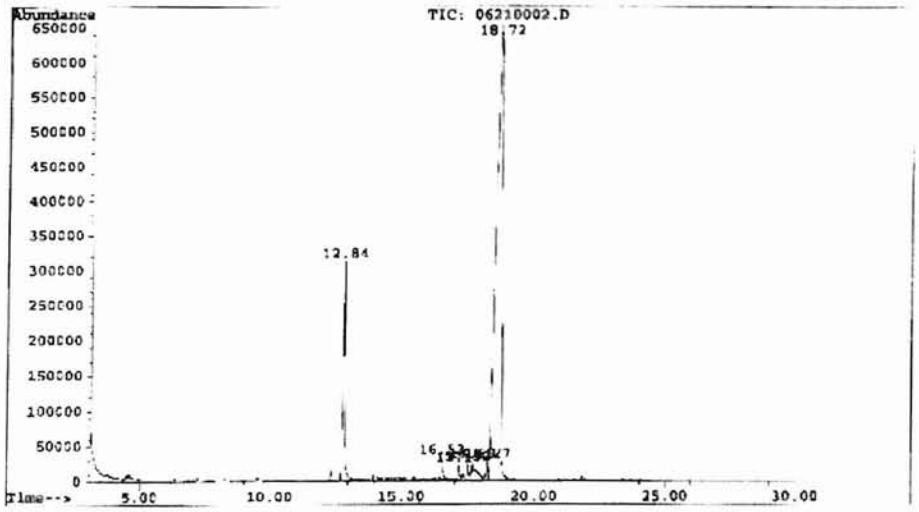
12.84 represents benzeneacetamide

Figure 13. GC/MS Results for B. bifidum 0 mM Phenobarbital



No peaks from parent compound (phenobarbital) or metabolite (benzeneacetamide) were detected.

Figure 14. GC/MS Results for *B. bifidum* 5 mM Phenobarbital

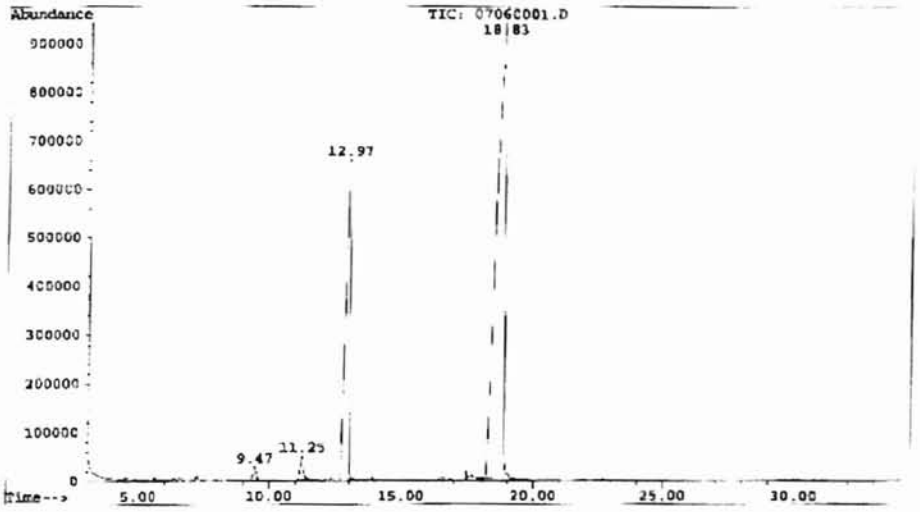


Major Peak Identification (in minutes)

18.72 represents phenobarbital

12.84 represents benzeneacetamide

Figure 15. GC/MS Results for *B. bifidum* 10 mM Phenobarbital

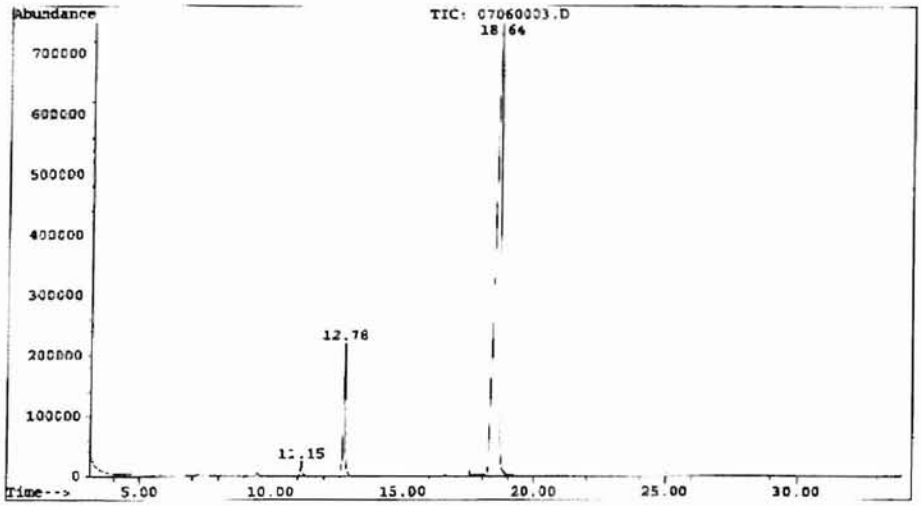


Major Peak Identification (in minutes)

18.83 represents phenobarbital

12.97 represents benzeneacetamide

Figure 16. GC/MS Results for *B. bifidum* 20 mM Phenobarbital



Major Peak Identification (in minutes)

18.64 represents phenobarbital

12.78 represents benzeneacetamide

Figure 17. Ratio of Benzeneacetamide to Phenobarbital versus Concentration

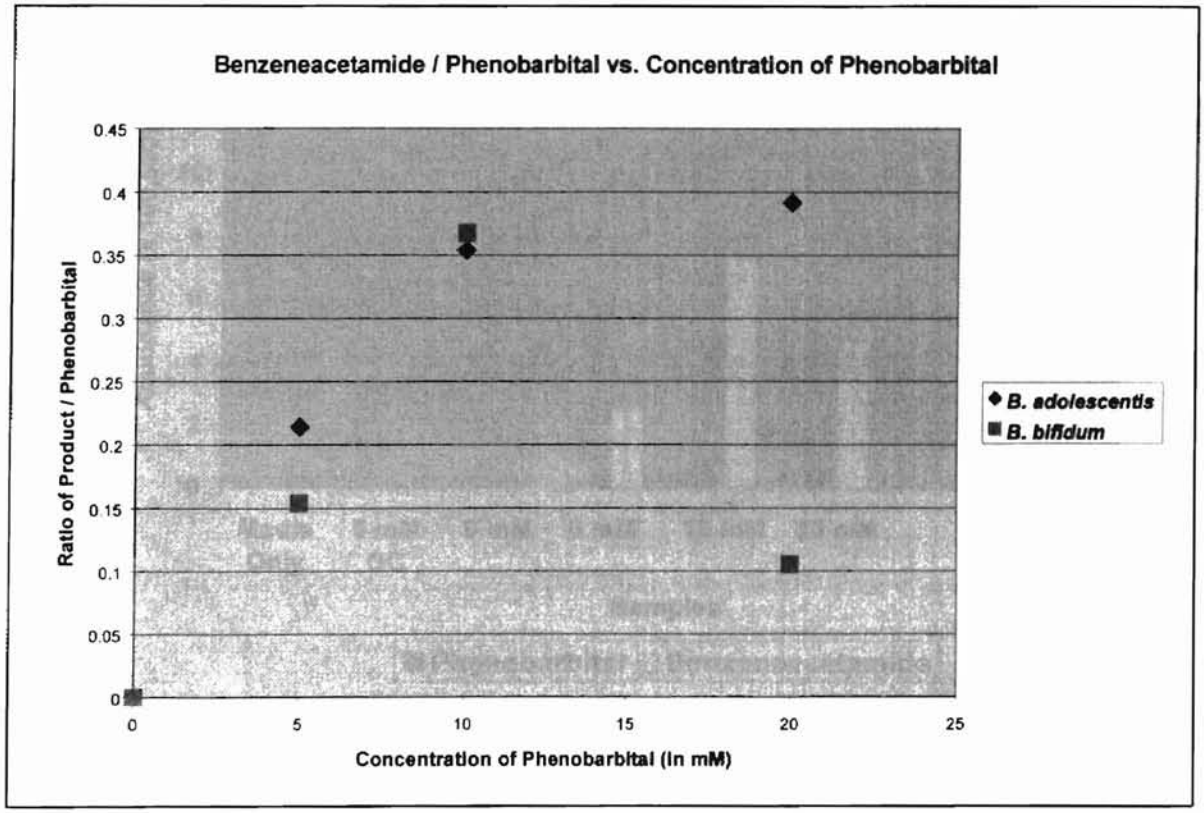
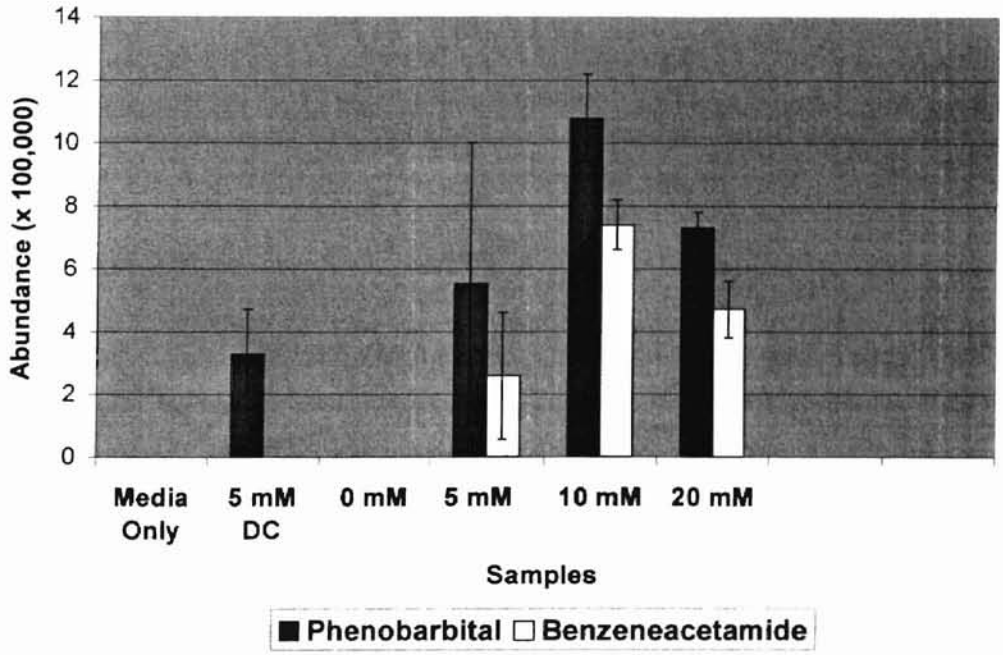
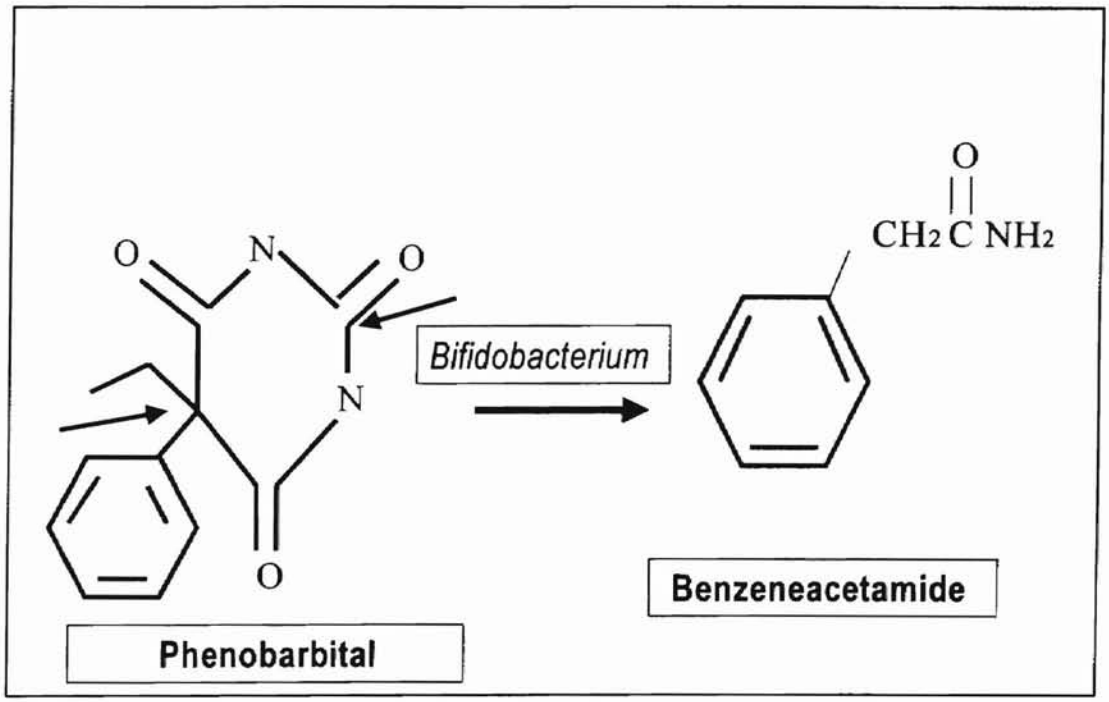


Figure 18. Metabolism of Phenobarbital from *B. adolescentis*



Media Only represents a control sample with no phenobarbital or bacteria addition. The 5 mM DC is a degradation control containing phenobarbital and no bacteria. Phenobarbital is shown in black and benzeneacetamide is shown in white (clear bars).

Figure 19. The Structure of Phenobarbital



CHAPTER 4

CONCLUSIONS AND DISCUSSION

This work began with the hypothesis that intestinal flora may contain a cytochrome P-450-like protein, in which to utilize for the metabolism of xenobiotics, similar to its eukaryotic partner located primarily in the liver. Phenobarbital was selected for experimentation because it is a compound that has been routinely used in the laboratory for the induction of cytochrome P450 complexes. Previous research has shown that phenobarbital is a strong inducer of specific cytochrome P-450 complexes in mammals. Following experimentation, it was determined that the chosen intestinal microorganism, *Bifidobacterium*, did not contain such an enzyme system. This was demonstrated by the lack of a 45 to 50 kDa protein induction in the SDS-PAGE and Silver Stain gels. Next, the absence of the cytochrome P450 characteristic peak at 450 nm made it evident that a P450 complex was not present in *Bifidobacterium*.

These findings led to several questions, including why this was occurring and what effect does the pharmaceutical, phenobarbital, have on the survival of the microorganism? It was the need to uncover the answer to these questions that led the researcher to proceed with the following experiments.

The second part of this project consisted of investigating what affect phenobarbital had on the growth of the microorganism, *Bifidobacterium*. Growth curve experiments were performed. The results of this experiment demonstrated an increase in doubling time as the concentration of phenobarbital was increased. Next, the growth curve samples underwent GC/MS evaluation to determine if phenobarbital was being metabolized in the presence of the microorganism, *Bifidobacterium*. The MS identified

two peaks as phenobarbital and benzeneacetamide. In contrast, the 0mM phenobarbital blank, 5 mM phenobarbital degradation control (no bacteria) and sterile media showed no major peaks. In the presence of *Bifidobacterium*, phenobarbital had undergone reductive cleavage as the terminal nitrogen obtained a hydrogen atom. It appeared that the phenobarbital was cleaved at three positions, releasing the CH_3CH_2 and CONCO molecules. The unique finding of this study showed for the first time how *Bifidobacterium*, a major intestinal microorganism, was capable of metabolizing phenobarbital into benzeneacetamide.

One explanation as to why phenobarbital metabolized by *Bifidobacterium* can best be described by the lac operon system in *E. coli* to utilize lactose as a carbon source. The lactose (*lac*) system is still the best-studied example of gene regulation. The mechanism of regulation of gene expression is today one of the most actively studied problems in molecular biology, in good part as a result of the pioneering work of Jacob and Monod on the control of genes involved in lactose metabolism in the bacterium, *E. coli* (3, 4, 29). In *E. coli*, two proteins are necessary for the metabolism of lactose: the enzyme B-galactosidase, which cleaves the disaccharide lactose (a B-galactoside) into the monosaccharides galactose and glucose, and lactose permease, a protein required for transport of lactose across the cell membrane into a cell(26). The regulatory mechanism of the lac system has many features. First, the lactose-utilization system consists of two kinds of components; structural genes needed for transport and metabolism of lactose and regulatory elements, such as the lacI gene, lacO operator, and the lac promotor. The lac operon system is inducible and lactose is the inducer(4, 20, 26). It is possible that the delay in lag phase we have seen in our growth curve experiments is due to the presence

of an inducer, in our case, phenobarbital, that activates a 'hypothetical' phenobarbital operon. This phenobarbital operon may be responsible for the production of biological elements, such as drug metabolizing proteins that have the ability to utilize phenobarbital.

Our results have clinical significance regarding the role of intestinal flora in the metabolism of foreign compounds, such as phenobarbital. Our results have demonstrated that *Bifidobacterium* can metabolize phenobarbital to a potentially toxic substance, benzeneacetamide. Because intestinal flora possess a diverse metabolic system, future studies should include more pharmaceutical testing prior to drug approval.

Future work in this area would be to see if the same metabolic phenomenon exists in other intestinal microorganisms. The intestinal tract, and the microorganisms that colonize it, is an area that has yet to be examined thoroughly. The comprehension of the degradation of xenobiotics by intestinal microorganisms is generally overlooked yet may provide insight to the understanding of human health and well-being.

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Kayleen R. Tucker

Education: B.S., Biology – UNIVERSITY OF NEBRASKA
Lincoln, Nebraska 68508
Cumulative GPA: 3.000
August 1994 to May 1998

M.S., Microbiology and Molecular Genetics – OKLAHOMA STATE UNIVERSITY
Stillwater, Oklahoma 74078
Cumulative GPA: 3.500
Completed the requirements for the M.S. degree at Oklahoma State University in December, 2000.

Organizations: Microbiology and Molecular Genetics Graduate Student Association
- President, 1999-2000

American Society of Microbiology
Omaha Nebraska Phi Mu Sorority Alumni Chapter
University of Nebraska Alumni Association

VITA

Kayleen R. Tucker

Candidate for the Degree of

Master of Science

Thesis: THE EFFECTS OF PHENOBARBITAL, A DRUG USED TO TREAT EPILEPSY, ON INTESTINAL FLORA, *BIFIDOBACTERIUM SP.*

Major Field: Microbiology, Cell and Molecular Biology

Biographical:

Personal Data: Born in Omaha, Nebraska, on Nov. 26, 1975, the daughter of James and Constance Tucker.

Education: Graduated from Bryan High School, Omaha, Nebraska in May 1994; received Bachelor of Science degree in Biological Sciences from the University of Nebraska, Lincoln, Nebraska in May 1998. Completed the requirements for the Master of Science degree with a major in Microbiology, Cell and Molecular Biology in December, 2000.

Experience: Employed as a pharmacy technician throughout enrollment at the University of Nebraska; employed by Oklahoma State University, Department of Microbiology as a teaching assistant, May 2000; hired as a Research Assistant I by the Department of Obstetrics and Gynecological Oncology at the University of Oklahoma Health Science Center, July 2000.

Professional Memberships: American Society of Microbiologists, Microbiology Graduate Student Association, University of Nebraska Alumni Association, and Huskerfever.