Metabolic Responses of Enterococcus faecium to Bisphenol A (an endocrine disruptor chemical)

A Thesis Submitted in partial fulfillment of the requirements for the

Degree of

Master of Science in Biotechnology

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Candidate's Declaration

I hereby declare that the work presented in the dissertation entitled **"Metabolic responses of *Enterococcus faecium* to Bisphenol-A (an endocrine disrupting chemical)"** in partial fulfillment of the requirements for the degree of Master of Science in Biotechnology, written and submitted by me to Thapar University, Patiala is an authentic record of my own work during the period of 6 months from January 2011 to June 2011 under the supervision of Dr. Moushumi Ghosh, Assistant Professor, Thapar University, Patiala.

Date: 18 July, 2011

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Certificate

This is to certify that the thesis entitled **"Metabolic Responses of *Enterococcus faecium* to Bisphenol A (an endocrine disrupting chemical)"** submitted by Sheetal Garg in partial fulfillment of the requirements for the award of Degree of Master of Science in Biotechnology to Thapar University, Patiala, is a record of student's own work carried out by her under my supervision and guidance. The report has not been submitted for the award of any other degree or certificate in this or any other University or Institute.

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List of Abbreviations

BPA	Bisphenol A
E.faecium	*Enterococcus faecium*
MRS	Man Rogosa de Sharpe media
EDC	Endocrine Disrupting Chemical
Cfu	Colony Forming Unit
SDS PAGE	Sodium dodycyl Sulphate Polyacrylamide gel electrophoresis
APS	Ammonium per sulphate
TEMED	Tetramethylethylenediamine
BSA	Bovine serum albumin
KD	Kilo Dalton
Mw	Molecular weight
rpm	Revolution per minute

List of Symbols

ml	Millilitre
µl	Microlitre
mg	Milligram
ng	Nanogram
nm	Nanometer
gm	Gram
L	Litre
M	Molar
h	Hour
h^{-1}	Per hour
V	Volt
A	Ampere
°F	Degree fahrenheit
%	Percent
µg/kg	Microgram per kilogram
ng/Kg	Nanogram per kilogram
µg/ml	Microgram per Millilitre
w/v	Weight by volume
v/v	Volume by volume
K_2HPO_4	Dipotassium hydrogen phosphate
KCl	Potassium chloride

Formula	Name
$MgSO_4$	Magnesium chloride
$MnSO_4$	Manganese sulphate
$Na_2HPO_4.7H_2O$	Disodium hydrogen phosphate
NaCl	Sodium chloride
NH_4Cl	Ammonium chloride
$CaCl_2$	Calcium chloride
$NaHCO_3$	Sodium bicarbonate

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Abstract

Bisphenol A (BPA), a putative endocrine disruptor, may be taken up by humans via the diet and have adverse effects on human health. In this study, we evaluated the response of *E.faecium,* predominant lactic acid bacteria of the intestine, to Bisphenol A, a constituent of epoxy and polystyrene resins that is used extensively in the food packaging industry and dentistry. Significant reduction in log cfu of *E.faecium* i.e. 32% and 42.5% was observed in the media containing 40µg/ml and 50µg/ml of Bisphenol A, respectively. Further Bisphenol A treated *E.faecium* was studied for its survival after passage through artificial saliva, gastric and intestinal juice and was found to be tolerant to gastric and intestinal transit. Also, since adhesion is an important attribute for gut intrinsic flora to exert their beneficial effects, BATH test was conducted to observe the change in cell surface hydrophobicity of Bisphenol A treated *E.faecium* compared to untreated *E.faecium.*

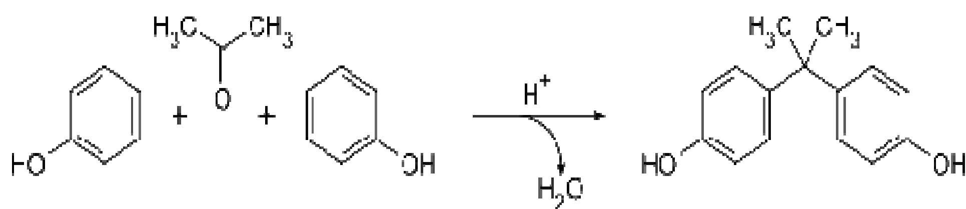
The human intestine houses a large number of bacteria belonging to 300–500 different species which live and grow as symbionts. The stomach, duodenum (0-10^4 bacteria/g of the luminal contents) and small intestine (10^5-10^6 bacteria/g) contain smaller number of bacteria adhering to the epithelia and some other bacteria in transit. This may be because of the composition of the luminal fluid containing acid, bile, and pancreatic secretion, which kill most ingested microorganisms. On the other hand, the large intestine contains a complex and dynamic microbial population with high density of living bacteria. The luminal contents may have up to 10^{11} or 10^{12} bacteria/g. Some of these bacteria are potential pathogens and can be a source of infection and sepsis under certain conditions, for example when the integrity of the bowel barrier is physically or functionally broken down.

Microorganisms start colonization of the gastrointestinal tract soon after birth and this process continues throughout the life. The environmental factors have a major role in determining the extent and type of some bacteria can modulate expression of genes in host epithelial cells, thus creating a favourable habitat for themselves, and can prevent growth of other bacteria introduced later. The initial colonization is therefore very relevant to the final composition of the permanent flora in adults. The predominant genera in human beings are *Bacteroides, Bifidobacterium*, *Eubacterium*, *Clostridium*, *Peptococcus*, *Peptostreptococcus*, and *Ruminococcus etc*. followed by aerobes (facultative anaerobes) such as *Escherichia, Enterobacter, Enterococcus, Klebsiella, Lactobacillus, Proteus etc*. Every individual has several hundreds of species, with a particular combination that is distinct from that found in other individuals. The gastrointestinal tract (GIT)

is exposed to different environmental pollutants that contaminate food and water. These include chemicals with an ability to disrupt the endocrine system. The body has developed various mechanisms to detoxify the toxic substances, including metals and chemicals. The gut resident microbes are important in this detoxification process. However little is known regarding their behavior upon exposure to such toxic chemicals. In vitro studies using selected gut flora and toxic chemicals may be helpful in elucidating the role of such bacteria in detoxification inside the gut. One frequently studied gut bacterium, *E.faecium*, resides mainly in Peyer's patches and the small intestine and is the predominant bacterial species in the intestines of breast-fed infants, where it presumably prevents colonization by potential pathogens. Healthy infants tend to display higher levels of *E.faecium* than do infants with allergies, suggesting a correlation between allergic disease and intestinal *E. faecium* flora that have reduced adhesive abilities to the intestinal mucosa. If so, this may be influential to human health, since disruption of the normal microbiota mediated mechanism of immunomodulation in the mucosa leads to an increase in theincidence of allergic disease or other gastrointestinal (GI) disease.

Thus *E.faecium* assumes considerable relevance as a model microorganism for investigating effects of toxic chemicals ingested regularly through food and water. One such chemical compound is Bisphenol A, to which human beings are exposed on a daily basis through consumption of food and beverages as well as through environmental routes. Bisphenol A (BPA) is a high volume production chemical used to make epoxy resin and polycarbonate plastic products, including some kinds of water bottles, baby bottles, and food storage and heating containers. Polycarbonate plastic can become unstable over time and with use, allowing Bisphenol A to leach into material in contact with the plastic. Additionally, Bisphenol A is now pervasive in the environment and commonly found in dust particles, surface water and drinking

water, as over 6 billion pounds are produced worldwide each year and production of Bisphenol A releases approximately 2 hundred thousand pounds of the chemical into the atmosphere annually. Bisphenol A was first synthesized by the Russian chemist A.P. Dianin in 1890, the compound is synthesized by the condensation of acetone with two equivalents of phenol. The reaction is catalyzed by a strong acid, such as hydrogen chloride (HCl) or a sulfonated polystyrene resin. Industrially, a large excess of phenol is used to ensure full condensation, the product mixture of the cumene process (acetone and phenol) may also be used as starting material.



The extensive production range of BPA in consumer products is due to its excellent physical and chemical properties. BPA's polymer, polycarbonate, has the unique ability to combine toughness, good transparency, and elevated mechanical strength into a lightweight and high performance plastic. However, the health and environmental concerns arose because the polycarbonate is not stable when conditions such as heat, bases or acids are present. This volatility is due to the linking bonds (ester bonds) between BPA molecules, which are easily broken down under such conditions. This results in a high probability that BPA will leach from the plastic into the body and the environment. The BPA leached from the plastics into food and water which is then consumed by humans can lead to disruptions associated with endocrine hormones.

Bisphenol A mimics estrogen activity and is known as an "endocrine disruptor", a chemical that interferes with the hormonal system in animals and humans and contributes to adverse health

effects. Bisphenol A causes a variety of impacts through mechanisms of action that are probably unrelated to estrogenic properties. The link of BPA with infertility is unequivocal. Those most vulnerable to problems resulting from BPA exposure are pregnant mothers and newborns. BPA has been detected in various fluids such as amniotic fluid, maternal and fetal plasma, placenta, and breast milk. It may be concluded that BPA, at chronic and subchronic levels, alters several variables: embryonic development, hatching, body growth, and reproductive maturation. Ramakrishna and Wayne (2007) observed that growth patterns of the organisms consistently show undersized patterns when compared to control groups. Furthermore, changes in the onset of puberty and in reproductive functioning have been observed which is consistent with many previous studies. Although a vast gamut of information is currently available on the effects of Bisphenol A in human physiology and metabolism, not much information regarding the behavior of human gut flora to Bisphenol A is available. It was therefore envisaged to evaluate the behavior of *E.faecium* to Bisphenol A, following objectives were framed.

1. To study the effect on survival of *E.faecium* under exposure to various Bisphenol A levels
2. To examine whether Bisphenol A treated *E.faecium* lose or retains its functionality in terms of human benefits.

Esophagus, stomach, duodenum, small intestine (ileum), large intestine (colon), appendix, liver, gallbladder and pancreas all comprise a gut by forming a tube connection between mouth and anus through which food passes. GI tract (GIT) is a tubular passage made of muscle and mucous membrane that extends about 8.3 meters in length. Colon, a tube-like organ is made of 4 sections: the ascending colon, transverse colon, the descending colon, the sigmoid colon and it is about 1.5 to 2 meters long. Its primary function is to absorb water and salts from undigested foods and store the waste-products until excreted. The colon is viewed as the preferred absorption site for oral administration of protein and peptide drugs because of the relatively low proteolytic enzyme activities in the colon (Yang *et al*., 2002).

The flora of the GIT in mammals is highly complex and diverse. The normal intestinal immune system is under a balance in which proinflammatory and anti- inflammatory cells and molecules are carefully regulated to promote a normal host mucosal defense capability without destruction of intestinal tissue (Hahm *et al*., 2001). Once this careful regulatory balance is disturbed, nonspecific stimulation and activation can lead to increased amounts of potent destructive immunological conditions. It is imperative that any perturbation of the intestinal barrier may lead to promotion of bacterial adherence. A disturbance in distribution of gut inhabiting microbes due to antibiotics or toxic chemicals or metals can completely disrupt the fine balance of synergy between the human body and the microbes.

Endocrine disruptors are man-made synthetic chemicals and natural phytoestrogens (naturally occurring plant or fungal metabolite derived estrogen) that act on the endocrine systems of

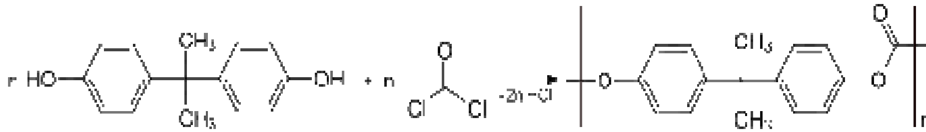
humans and animals by mimicking, blocking and or interfering in some manner with the natural instructions of hormones to cells. In other words, endocrine disruptors are an exogenous agent that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body which are responsible for the maintenance of homeostasis, reproduction, development, and behaviour.

Webster's defines exogenous as something which is introduced from or produced outside the organism or system; specifically not synthesized within the organism or system. The bodies of humans and other animals depend upon a complexly integrated and timed series of events, of which the delivery of hormones to various organs is vital. When the delivery timing and amount of a hormone are upset the results can be devastating and permanent. The disruption can take place as an inappropriate quantity or timing of a response to a stimulus, the blocking of hormonal effects in parts of the body normally sensitive to it, and the stimulation or inhibition of the endocrine system that could produce an inappropriate quantity of hormones — too much, too little or none at all.

Any combination of these interferences on the endocrine system can affect physical development, sex, reproduction, brain development, behavior, temperature regulation and more. An endocrine disruptor can injure or destroy an organ that has the task of supplying hormones. Several evidences present that endocrine disruptors have effects on male and female reproduction, breast development and cancer, prostate cancer, neuroendocrinology, thyroid, metabolism and obesity and cardiovascular endocrinology. Mechanisms by which endocrine disruptors exert their effect have grown. Endocrine-disrupting chemicals (EDCs) were originally thought to exert actions primarily through nuclear hormone receptors, including estrogen receptors (ERs), androgen receptors (ARs), progesterone receptors, thyroid receptors (TRs), and

retinoid receptors, among others. Thus, endocrine disruptors act via nuclear receptors, nonnuclear steroid hormone receptors (*e.g*., membrane ERs), non steroid receptors (*e.g*., neurotransmitter receptors such as the serotonin receptor, dopamine receptor, norepinephrine receptor), orphan receptors (*e.g*., aryl hydrocarbon receptor (ahr)—an orphan receptor), enzymatic pathways involved in steroid biosynthesis and/or metabolism, and numerous other mechanisms that converge upon endocrine and reproductive systems. Thus, from a physiological perspective, an endocrine disrupting substance is a compound, either natural or synthetic, which through environmental or inappropriate developmental exposures, alters the hormonal and homeostatic systems that enable the organism to communicate with and respond to its environment. Natural chemicals found in human and animal food (*e.g*., phytoestrogens, including genistein and coumestrol) can also act as endocrine disruptors. These substances, whereas generally thought to have relatively low binding affinity, are widely consumed and are components of infant formula.

The group of molecules identified as endocrine disruptors is highly heterogeneous and includes synthetic chemicals used as industrial solvents/lubricants and their byproducts (polychlorinated biphenyls (pcbs), polybrominated biphenyls (pbbs), dioxins), plastics (Bisphenol A (BPA), plasticizers (phthalates)), pesticides [methoxychlor, chlorpyrifos, dichlorodiphenyltrichloroethane (DDT)], fungicides (vinclozolin), and pharmaceutical agents (diethylstilbestrol). Bisphenol A is used primarily to make plastics, and products containing Bisphenol A based plastics have been in commercial use since 1957. At least 8 billion pounds of BPA are used by manufacturers yearly. It is a key monomer in production of epoxy resins (Replogle and Jill, 2009) and in the most common form of polycarbonate plastic (Kroschwitz, Jacqueline, 2009). The overall reaction to give polycarbonate can be written:



Polycarbonate plastic, which is clear and nearly shatter-proof, is used to make a variety of common products including baby and water bottles, sports equipment, medical and dental devices, dental fillings and sealants, eyeglass lenses, CDs and DVDs, and household electronics. BPA is also used in the synthesis of poly sulfones and polyether ketones. It is used as an antioxidant in some plasticizers, and as a polymerization inhibitor in PVC. Epoxy resins containing Bisphenol A are used as coatings on the inside of almost all food and beverage cans (Erickson, Britt E., 2008 however, due to BPA health concerns, in Japan epoxy coating was mostly replaced by) PET film (Byrne and Jane, 2008). Bisphenol A is also a precursor to the flame retardant tetrabromobisphenol A, and was formerly used as a fungicide. Bisphenol A is a preferred color developer in carbonless copy paper and thermal paper with the most common public exposure coming from some thermal point of sale receipt paper (Raloff and Janet, 2009). BPA based products are also used in foundry castings and for lining water pipes "In general, plastics that are marked with recycle codes 1, 2, 4, 5, and 6 are very unlikely to contain BPA.

Some, but not all, plastics that are marked with recycle codes 3 or 7 may be made with BPA (Fiege *et al*., 2002). Worldwide production of Bisphenol A exceeds six billion pounds per year and demand for the chemical has risen significantly in recent years. Exposure to Bisphenol A is widespread. The Centers for Disease Control and Prevention (CDC) found that 95 percent of Americans tested have detectable levels of BPA in their bodies and a 2008 study shows that BPA levels are lowest in adults, mid-range in adolescents and highest in children. What cannot be disputed is that a number of tests have detected Bisphenol A in human tissue and this indicates widespread exposure. Coupled with the knowledge of increasing rates of infertility, prostate and

breast cancer and other conditions such as diabetes and obesity in the human population, the possible effects of hormone disruptors like Bisphenol A cannot be ignored. Repeated washing of the bottles (approximately 60–100 washes) indicates the quantity of Bisphenol A that would leach from a well-used bottle. Although it is higher than the temperature range of domestic dishwashers, heating to 80ºC/176ºF represents the effect of repeated scrubbing and exposure to detergents and warm water.

Water in glass bottles, either untreated or spiked with BPA (8.5ng/ml), were run as negative and positive controls, respectively. The bottles did not show notable levels of leaching at room temperature but all bottles showed significant levels of leaching in the range of 5-8ng/ml (ppb) when heated. All reported values of 0.00 ng/ml indicate concentrations that could not be detected or fell below the limit of detection of the analytical equipment. Repeat washing in the dishwasher, or simply with hot water, the plastic can degrade and the amount of Bisphenol A leaching from the bottle increases. Scientific studies have simulated repeated and long-term use of polycarbonate bottles and found Bisphenol A continues to leach over time. This indicates that polycarbonate plastic products degrade over time and with normal use, especially after exposure to heat. Heated to 80°C to simulate 60-100 washings and the effect of detergent on polycarbonate plastics, Baby's Toxic Bottle confirms these previous findings.

Acidic materials, such as apple juice, break apart the bonds that hold Bisphenol A molecules together. The chemical ester bonds in Bisphenol A interact with the free hydrogen in acids and break apart. Bisphenol A is primarily hydrophobic, meaning it does not easily dissolve in water but is more soluble in alcohol or fatty materials. Given this characteristic, Bisphenol A is likely to leach more in milk than in water. Given the extensive peer-reviewed literature on BPA

showing adverse effects in animals at low levels it may be concluded that the amount leaching from heated bottles is a potential health risk for infants drinking from them.

Bisphenol A is an endocrine disruptor, which can mimic the body's own hormones and may lead to negative health effects. Early development appears to be the period of greatest sensitivity to its effects. Regulatory bodies have determined safety levels for humans, but those safety levels are currently being questioned or under review as a result of new scientific studies. Recent studies indicated that "perinatal BPA exposure acts to exert persistent effects on body weight and adiposity" (Soto, 2009). Also low doses of BPA during development have persistent effects on brain structure, function and behavior in rats and mice (Richter and Birnbaum, 2007). Neonatal exposure to Bisphenol A (BPA) can affect sexually dimorphic brain morphology and neuronal adult phenotypes in mice (Patisaul and Polston, 2008).

A study with rats prenatally exposed to 40μg/kg BPA has shown that corticosterone and its actions in the brain are sensitive to the programming effects of BPA (Poimenova and Markaki, 2010). It has also been concluded that BPA mimics estrogenic activity and impacts various dopaminergic processes to enhance mesolimbic dopamine activity resulting in hyperactivity, attention deficits, and a heightened sensitivity to drugs of abuse (Jones and Miller, 2008). Another study on rats has concluded that prenatal and neonatal exposure to low dose BPA causes deficits in development at dorsolateral striatum via altering the function of dopaminergic receptors (Hou and Zhang, 2009). It has been concluded that Bisphenol A has been shown to bind to thyroid hormone receptor and perhaps have selective effects on its functions (Zoeller, 2007). Another review about environmental chemicals and thyroid function, raised concerns about BPA effects on triiodothyronine and concluded that agencies need to regulate the use of thyroid-disrupting chemicals, particularly as such uses relate exposures of pregnant women,

neonates and small children to the agents" (Boas and Main, 2009). BPA is able to induce neoplastic transformation in human breast epithelial cells, besides maternal oral exposure to low concentrations of BPA during lactation increases mammary carcinogenesis in a rodent model.

The mammary glands of the offspring of pregnant rats treated orally with 0, 25 or 250µg BPA/kg body weight has found that key proteins involved in signaling pathways such as cellular proliferation were regulated at the protein level by BPA. A study in mice has found that neonatal BPA exposure at 10µg/kg disrupted the development of the fetal mouse prostate (Richter *et al.,* 2005*)*. In vitro study has found that BPA within the range of concentrations currently measured in human serum is associated with permanently increase in prostate size (Richter *et al.*, 2007). Newborn rats exposed to a low dose of BPA (10µg/kg) increased prostate cancer susceptibility when adults (Prins *et al.*, 2009), Mouse ovary anomalies from exposure as low as 1µg/kg, concluded that BPA exposure causes long-term adverse reproductive and carcinogenic effects if exposure occurs during prenatal critical periods of differentiation. Neonatal exposure of as low as 50µg/kg disrupts ovarian development in mice, besides BPA as low as 50µg/kg permanently alters the hypothalamic estrogen dependent mechanisms that govern sexual behavior in the adult female rat (Monje *et al.*, 2009).

It has been concluded that BPA, at the reference safe limit for human exposure, was found to impact intestinal permeability and may represent a risk factor in female offspring for developing severe colonic inflammation in adulthood (Braniste *et al.,* 2009). A 2010 study on mice has concluded that perinatal exposure to 10µg/ml of BPA in drinking water enhances allergic sensitization and bronchial inflammation (Midoro-Horiuti *et al.,* 2010*)*. The impact of endocrine disrupting chemical (EDC) exposure on human health is receiving increasingly focused attention. The prototypical EDC Bisphenol A (BPA) is an estrogenic high-production chemical used

primarily as a monomer for production of polycarbonate and epoxy resins. It is now well established that there is ubiquitous human exposure to BPA. In the general population exposure to BPA occurs mainly by consumption of contaminated foods and beverages that have contacted epoxy resins or polycarbonate plastics (Kang *et al.,* 2008). To test the hypothesis that bioactive BPA was released from polycarbonate bottles used for consumption of water and other beverages, they evaluated whether BPA migrated into water stored in new or used high-quality polycarbonate bottles used by consumers. Using a sensitive and quantitative competitive enzyme-linked immunosorbent assay, BPA was found to migrate from polycarbonate water bottles at rates ranging from 0.20 to 0.79ng per hour. At room temperature the migration of BPA was independent of whether or not the bottle had been previously used. Exposure to boiling water (100°C) increased the rate of BPA migration by up to 55-fold. The estrogenic bioactivity of the BPA like immunoreactivity released into the water samples was confirmed using an *in vitro* assay of rapid estrogen-signaling and neurotoxicity in developing cerebellar neurons. (Belche*r et al.,* 2005*)*

3.1 Media and chemical used

MRS medium (enriched medium) and M9 medium were used for culturing bacteria. Bisphenol A was purchased from Sigma (Mo, USA) and used with as per MSDS recommendation. The water solubility of Bisphenol A is moderate i.e.120-300mg in one liter. Accordingly, the stock of 30mg in 100ml water was prepared and then from this stock, working concentrations of 2.5µg/ml, 5µg/ml, and 10-50µg/ml were prepared by applying stoichiometric equation.

3.2 Bacterial culture and culture conditions

Growth conditions*:* *E.faecium* strain, an infant gut isolate used in this study was isolated and characterized previously in this laboratory (Sood, 2010). For routine use, the strain was individually sub cultured from glycerol stocks at -80° C in 5ml MRS broth and was grown at 37° C. For assays with freshly grown bacteria, cells were cultured in MRS broth for 18h, at 37° C.

3.3 Visual detection of Bisphenol A toxicity to *E. faecium*

From overnight grown culture of *E.faecium*, 100µl was spread on agar plates. Media plates were divided into four equal parts. A disc was placed in each part. 20µl of different concentrations of Bisphenol A (10µg/ml, 20µg/ml, 30µg/ml, 40µg/ml and 50µg/ml) was loaded onto the discs. Plates were incubated at 37°C for 18-24 hrs and zone of inhibition recorded.

3.4 Survival of *E.faecium* to different concentrations of Bisphenol A

3.4.1 High throughput survival analysis

Overnight grown culture of *E.faecium* was reinoculated into fresh 10ml MRS broth, incubated at 37°C, till log phase was achieved (4 hours) following which, its absorbance was noted at 600nm. The culture was then diluted to 0.03 of the absorbance value. Different concentrations (µg/ml) of BPA ranging from (2.5, 5, 10, 20, 30, 40, and 50) were prepared. Experiment was performed with each of the above concentrations being used in different 5ml MRS broths inoculated with 1ml of the diluted bacterial culture. From this, 200µl of each prepared sample was transferred into 96 well microtiter plate which was then put on high throughput growth analysis (Bioscreen Helsinki, Finland) system for studying the survival of bacterial culture to different concentrations of BPA. Absorbance was automatically recorded at 600nm for 24 hours at a time interval of 30 minutes.

3.4.2 Viability

Overnight cultures of *E.faecium* in the MRS broth were incubated at 37°C for 24 h for optimal growth. 1 percent (v/v) was reinoculated into 100ml MRS broth. Tenfold serial dilutions from 10^{-1} to 10^{-10} bacterial cultures were made when log phase was achieved (4 hours). Then, 10^{-7} to 10^{-10} diluted bacterial suspensions 0.1ml were spread plated onto an MRS agar plate and incubated for 24 hours for the colony counts. The effective range of colony counts were calculated as 25 to 250 colony - forming units (cfu)/per plate.

When log phase of culture was achieved, Bisphenol A was added into 100ml MRS broth at final concentrations of 40µg/ml and 50µg/ml. After adding BPA, absorbance was noted after every 30 minutes and simultaneously 1ml was taken out, tenfold serial dilutions from 10^{-1} to 10^{-10}

bacterial cultures were made, 0.1ml broth was taken out from dilutions 10^{-7} to 10^{-10} MRS agar plates. The plates were incubated at 37°C for 24 hours and the growth of *E.faecium* was observed.

Calculated bacterial concentrations were mean of colony counts × dilution 10 fold (10 n) × 10/volume of MRS plate. The effective range of colony counts was calculated as 25-250 cfu/per plate. Controls comprised of cultures devoid of Bisphenol A.

3.5 Protein profiling of Bisphenol A exposed and unexposed Cells by SDS PAGE

3.5.1 Protein extraction

Overnight grown culture of *E.faecium* was centrifuged at 8000 rpm for 5 minutes. Cell pellets were washed twice with chilled PBS and resuspended in 25 µl of cracking buffer and 25µl of double distilled water. Vortexed and kept at 100°C in water bath for 5 minutes. Pellets were sheared with a 191/2 gauge syringe for 4 to 5 minutes and centrifuged. The clear supernatant following centrifugation was stored in fresh eppendorf for analysis by SDS PAGE.

3.5.2 Protein Estimation

Protein estimation was done prior to assay by Bradford method. Briefly, BSA stock solution (1mg/ml), Dye was prepared as 10mg coomassie blue G250, 5 ml of 95 percent ethanol, 10 ml of 85% phosphoric acid was used, mixed and diluted to 100 ml by distilled water.

3.5.3 SDS PAGE

Gel preparation and sample loading

Assembly was set and 12% resolving gel was poured in between the two glass plates and the gel was overlaid by water : isobutanol :: 1:1 solution. Gel was allowed to solidify for half an hour. After solidification gel 4% stacking gel was poured over the resolving gel and comb was inserted in the space between the two glass plates. After solidification the gel was placed in the tank buffer and combs were removed. The sample was pipette down in the well along with the protein marker and parameters were set as-voltage: 65V, current-120A and time-45 min. The voltage was increased to 120V (in resolving gel). After the run, the gel was stained with coomassie brilliant blue (R-250) for 3-4 hrs. The gel was then destained by changing the destaining solution several times and visualized for whole cell protein patterns.

3.6 Survival of Bisphenol A treated *E.faecium* following successive passages through artificial saliva, gastric and intestinal juices

Reconstituted skim milk (15% w/v, Merck) was inoculated with approximately 2×10^8 cfu of an overnight culture of *E.faecium*. A 1ml aliquot was removed, serially diluted in QSR (Quarter Strength Ringer's Solution) and spread-plated onto MRS agar to determine the cfu at time 0 hr. To simulate the dilution and possible hydrolysis of bacteria in the human oral cavity, the suspension was diluted 1:1in a sterile electrolyte solution containing 6.2g/l NaCl, 2.2 g/l KCl, 0.22g/l $CaCl_2$ and 1.2g/l $NaHCO_3$ to which lysozyme was added to a final concentration of 100ppm, and incubated for 5 min at 37°C. The sample was subsequently diluted 3:5 with an artificial gastric fluid, consisting of the electrolyte solution mentioned above adjusted at pH 2.5 and with 0.3% pepsin (w/v) added. If required, pH was readjusted to pH 2.5 with 5M HCl. After

1h of incubation at 37°C, another 1ml aliquot was removed, serially diluted in QSR and spread-plated onto MRS agar. To simulate the dilution in the small intestine, the remaining volume was diluted 1:4 using an artificial duodenal secretion (pH 7.2) consisting of 6.4 g/l $NaHCO_3$, 0.239g/l KCl, 1.28g/l NaCl, 0.5% bile salts (Oxgall, Merck, Darmstadt, Germany) and 0.1% pancreatin (Hi Media, Mumbai, India) (Rosenberg *et al*., 1980). One milliliter aliquots were removed after 2 and 3h, serially diluted in QSR and spread-plated onto MRS agar to determine the cfu. Both Bisphenol A treated and untreated *E.faecium* was evaluated parallely for survival studies.

3.7 Adhesion of Bisphenol A treated *E.faecium* to hydrocarbons

For bacterial adhesion to hydrocarbons (BATH) (Alander *et al*., 1997) bacterial cells were washed with PBS buffer and resuspended in the same buffer. Absorbance was adjusted to 0.25 ± 0.05 to standardize the number of bacteria (10^8cells/ml) at 600 nm. Then, equal proportions of viable bacterial suspension and solvent (xylene) were mixed by vortexing for 5 min. The 2-phase system appeared and the aqueous phase was removed after 1 h of incubation at room temperature and its absorbance was measured. Also, we analyzed the viability influence in the BATH test by means of mixing heat inactivated bacterial suspensions (98°C during 10 min) with xylene. Results were reported as a percentages from 3 replicates according to the formula BATH %=[(A_0 – A)/A_0] ×100, where A_0 and A are absorbance before and after mixing with xylene, respectively.

To clarify the possible inhibitory effects of Bisphenol A to *E.faecium*, both MRS and minimal media were used. The use of a rich and stringent media reveals the actual behavior of microorganisms under conditions of luxury and stress-a condition frequented by a resident of gut.

4.1 Growth Kinetics

Growth kinetics of *E.faecium* in MRS indicate a maximal absorbance of 1.7 after 9 hours of growth with a lag phase of 1.8 hrs. The specific growth rate in MRS is 1.96 h^{-1}.

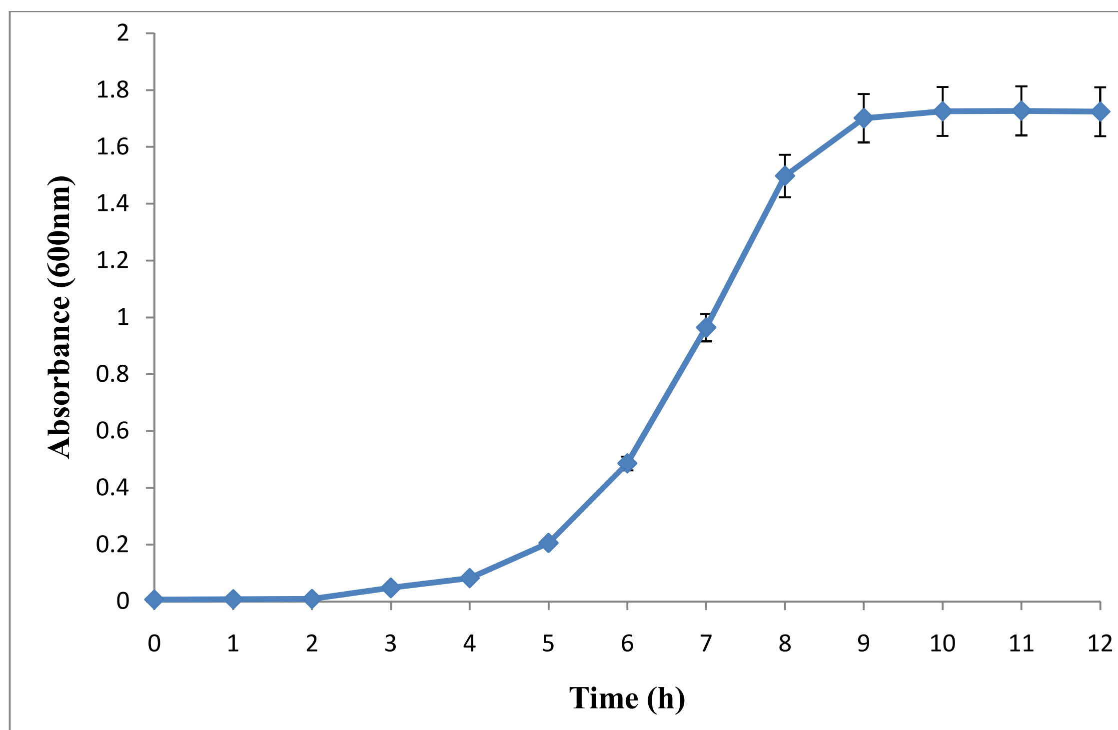


Fig1. Growth kinetics of Bisphenol A unexposed *E.faecium* in MRS media

4.2 Tolerance of *E.faecium* to Bisphenol A

Tolerance and growth of *E.faecium* towards Bisphenol A was studied in MRS broth containing different concentrations of Bisphenol A. Concentrations used were (2.5µg/ml, 5µg/ml, 10µg/ml, 20µg/ml, 30µg/ml, 40µg/ml, 50µg/ml). *E.faecium* was able to grow well at a concentration of 2.5µg/ml to 30µg/ml but at a concentration of 40µg/ml and 50µg/ml, where both growth rates was reduced significantly (Fig-2) an extended lag phase was predominant.

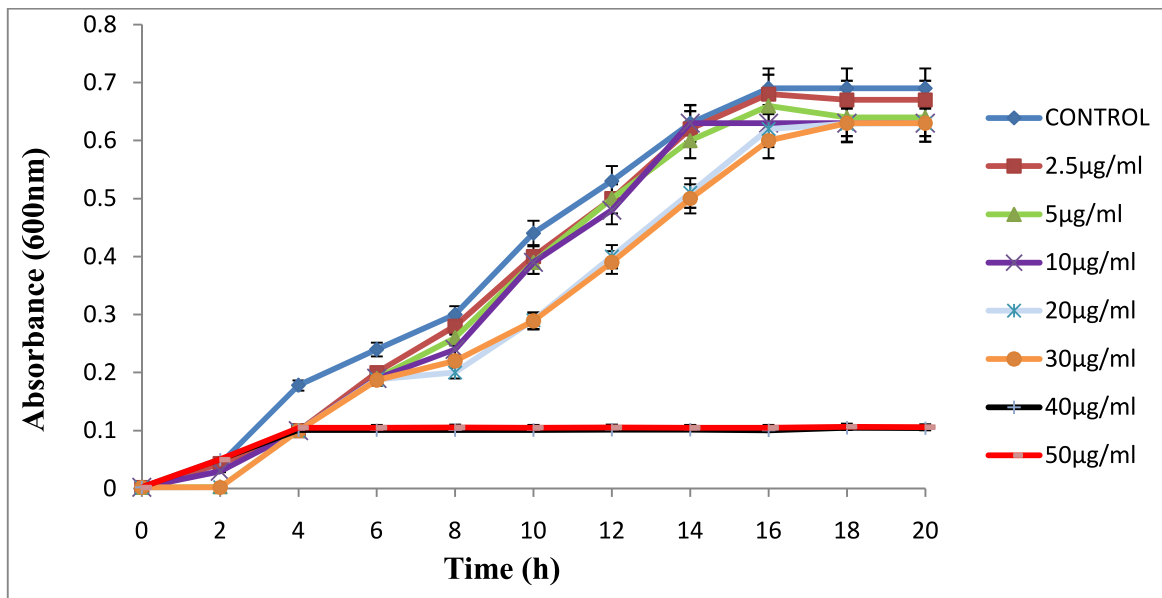


Fig.2 Kinetics of growth of *E.faecium* in MRS media with different concentrations of Bisphenol A

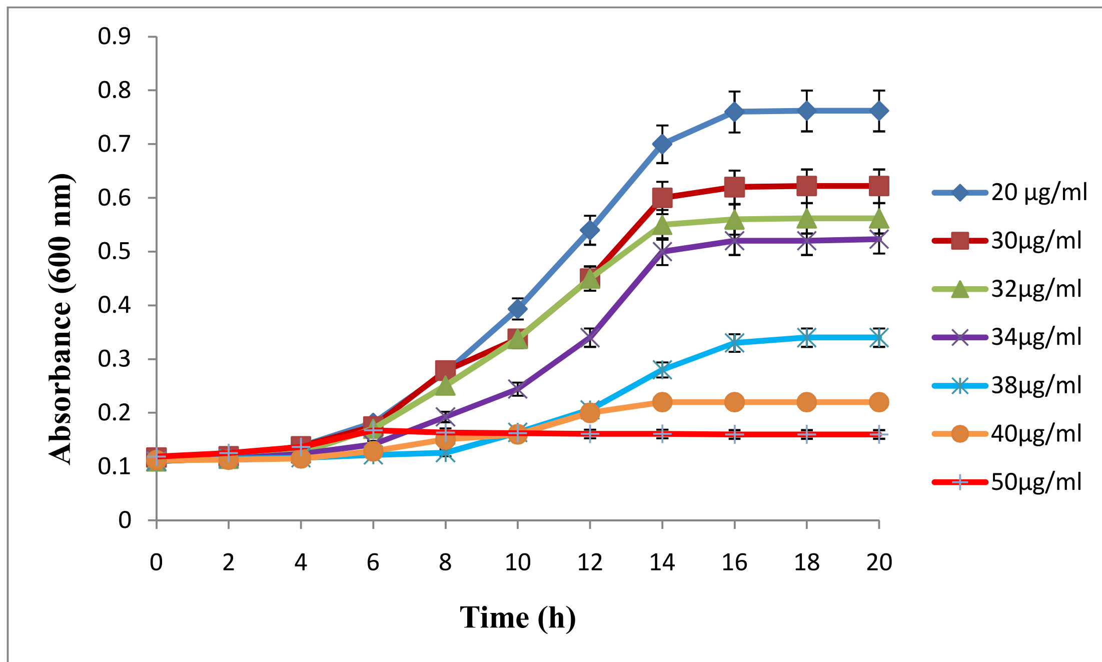


Fig.3 Growth Curve of *E.faecium* in M9 media (selective media) with different concentrations of Bisphenol A

In minimal media, the inhibitory effects of Bisphenol A were more extensive (Fig 3). This may be attributed to the presumable binding of Bisphenol A to media components in MRS or a resuscitating effect of MRS to toxicity of Bisphenol A to *E.faecium*. Such effects are not possible in minimal medium and leads to an enhanced toxicity of Bisphenol A to the E*.faecium*.

4.3 Growth of *E.faecium* with Bisphenol A

Notable differences in survival were evident at both 40µg/ml and 50µg/ml Bisphenol A. As much as 32.3% reduction in log cfu with 40µg/ml concentration of Bisphenol A and 42% reduction in log cfu with 50µg/ml concentration of Bisphenol A were recorded. It is clear from this and the above observations that Bisphenol A exerts an inhibitory effect to *E.faecium*.

Table 1: cfu and log cfu of *E.faecium* with 40µg/ml and 50µg/ml Concentration of Bisphenol A at different time intervals

Time (min)	cfu		Log cfu	
	40 µg/ml	50 µg/ml	40 µg/ml	50 µg/ml
0	1.87×1010	1.09×1010	10.02	10.00
20	1.02×1010	9.07×109	10.00	09.98
80	5.07×107	3.02×107	07.75	07.05
140	9.08×106	6.00×105	06.09	05.77

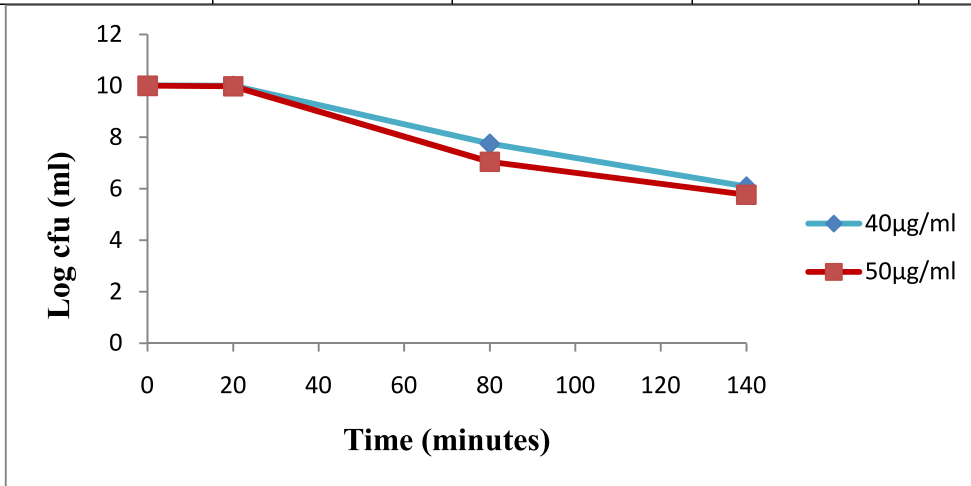


Fig.4Reduction in log cfu of *E.faecium* at 40µg/ml and 50µg/ml concentration of Bisphenol A

4.4 SDS PAGE

The whole cell protein profiles of Bisphenol exposed and unexposed *E.faecium* reveals (Fig. 4) the presence of at least two new polypeptides of Mw 21 KD and 14 KD. It is possible that these play an important role in protecting the cells from higher concentrations of Bisphenol A. However further studies are necessary for elucidating the importance of the polypeptides. A drastic disappearance of most of the proteins as compared to control *E.faecium* suggest that toxicity at the concentration used may have been alleviated by some mechanism which may be either degradation or binding or efflux from cells, this proposition needs further experiment substantiation.

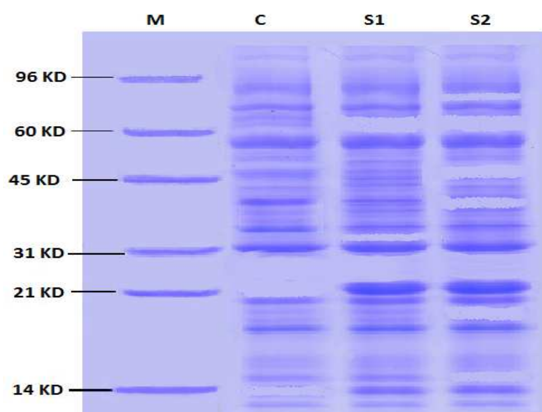


Fig. SDS PAGE of whole cell protein profile of *E.faecium* in response to Bisphenol A

Lane M- Molecular weight protein marker

C- Control; *E.faecium* without Bisphenol A exposure

S1- *E.faecium* exposed with 40µg/ml concentration of Bisphenol A

S2- *E.faecium* exposed with 50µg/ml concentration of Bisphenol A

4.5 Survival after the successive passages through artificial saliva, gastric and intestinal juices

Since stresses of stomach and small intestinal transit might interact synergistically, affecting the viability of the strains, it was deemed necessary to evaluate all components (enzymes, low pH, bile salts and food vehicle) in one system. The Bisphenol A treated *E.faecium* compared favorably to the untreated cells and was tolerant to the presence of lysozyme, acidic conditions (pH 2.5), 90 %, presence of pepsin and skimmed milk (15 % w/v), since in the various stages of incubation the counts did not change by more than approx. 0.5 log units (Fig.4).

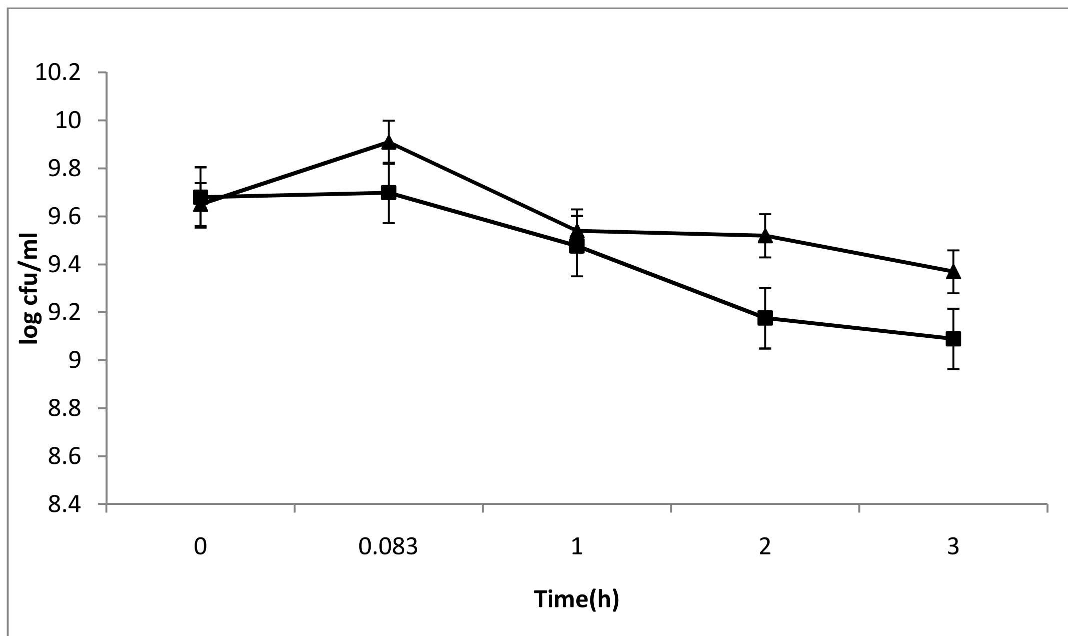


Fig.6 Effect of simulated gastric and intestinal transit on viability (log cfu) of Bisphenol A treated (■) and untreated *E.faecium* (▲).Values is the average from at least 3 experiments.

4.6 Adhesion to hydrocarbons

Adhesion is a complex trait that could be a multistep process in which both non-specific mechanisms and a specific ligand receptor play a role. The BATH test has been extensively used for measuring cell surface hydrophobicity in lactic acid bacteria (Gardiner *et al.*, 1999; Del Re *et al.*, 2000) and bifidobacteria (Reniero *et al.,* 1992) and is important attribute for gut intrinsic flora. The adhesion percentage of Bisphenol A treated and untreated *E.faecium* is shown in Table 2. The cell surface hydrophobicity decreased after Bisphenol A treatment between 7.35-49.5% units. The fact that a high percentage of cells following Bisphenol A treatment adhered to xylene, a polar solvent, indicated a hydrophobic cell surface of the *E. faecium* is retained following exposure.

Table 2: Adhesion to hydrocarbons of Bisphenol A treated and untreated viable *E. faecium* strain measured using the BATH test.

	% Adhesion to hydrocarbon (BATH Test)
Treatment	**Viable (mean ± s.d.)**
Untreated *E.faecium*	**72.87±11.0**
Bisphenol A treated *E.faecium*	**66.38±3.5**

4.7 Zone of Inhibition

Zone of inhibition was observed on MRS medium plates containing treated culture of *E.faecium* with 40µg/ml and 50µg/ml concentration of Bisphenol A. No zone of inhibition was observed in control and culture treated with 10µg/ml, 20µg/ml, and 30µg/ml concentrations of Bisphenol A.

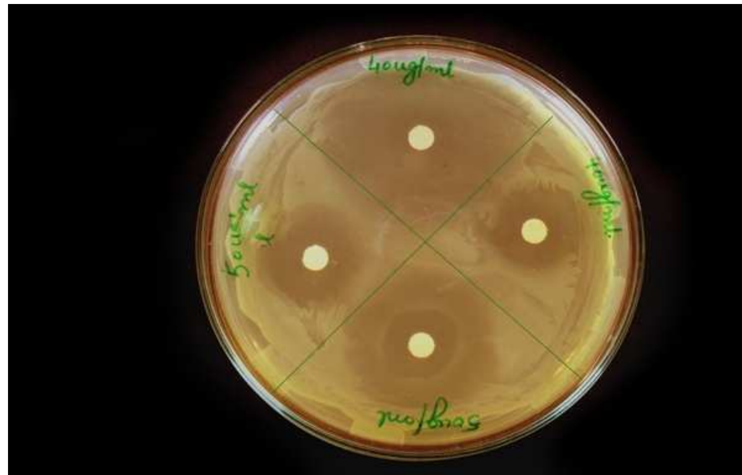


Fig.7 Plate showing zone of inhibition on MRS medium containing *E.faecium* treated with 40µg/ml and 50µg/ml concentration of Bisphenol A

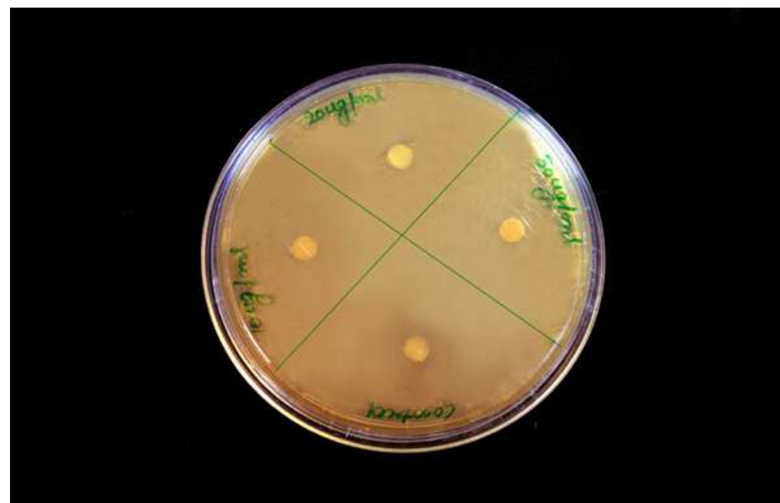


Fig.8 Plate showing no zone of inhibition on MRS medium containing untreated and treated *E.faecium* with 10µg/ml, 20µg/ml and 30µg/ml concentrations of Bisphenol A

Overall, the results of the above experiments indicate an inhibitory effect of Bisphenol A to the *E.faecium*, at concentrations of 40-50µg/ml. Although it is unlikely that such high concentrations of Bisphenol A would be encountered in reality by *E.faecium* cells, simulation under in vitro conditions may enable prediction of the behaviour especially in terms of the beneficial effects of this microorganism to human body under different concentrations of Bisphenol A ingestion through food contact materials or water. Since no earlier study similar to this exists, a comparison of the behavior of *E.faecium* during Bisphenol A exposure was not possible. The adhesive and hydrophobicity properties which are essential prerequisites for a gut bacteria to adhere and thus colonize the gut wall remain unaltered at the exposure levels used in this study thus implying that the cell surface and cell surface associated proteins responsible for binding to gut wall were not affected following exposure to Bisphenol A. The ability of the Bisphenol A exposed cells to survive gastric juice indicates its survival under the actual gut conditions. In order to further understand the effect of Bisphenol A on host beneficial effects of *E.faecium more* studies are mandatory.

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Man Rogosa de Sharpe (MRS) media:

S. No.	Chemical	g/l
1	Beef extract	10
2	Dextrose	20
3	K_2HPO_4	2
4	$MgSO_4$	0.1
5	$MnSO_4$	0.05
6	Protease peptone	10
7	Sodium acetate	5
8	Tri-Ammonium citrate	2
9	Yeast extract	5
10	Tween 80	1

Adjusted to 1000 ml with distilled water**.** Adjusted to pH 6.2 and sterilized by autoclaving.

M9 Minimal Media Composition (1000 ml)

M9 Salts Composition:-

Na_2HPO_4-$7H_2O$	64 g
KH_2PO_4	15 g
NaCl	2.5 g
NH_4Cl	5 g

Stirred until dissolved in 800 ml H_2O. Adjusted to 1000ml with distilled H_2O and sterilized by autoclaving.

M9 media Composition:-

M9 salts	200 ml
1M $MgSO_4$ (sterile)	2 ml
20% glucose (or other carbon source)	20 ml
1M $CaCl_2$ (sterile)	100 µl

Dissolved in ~700 ml sterile distilled water and adjusted to 1000ml with sterile distilled H_2O. Adjusted to pH 7.4

Chemical Requirements for SDS PAGE

Resolving Gel

Consisted of 3.3 ml water, 4 ml acrylamide solution, 2.5 ml Tris Buffer (1.5M) pH 8.8, 100 µl of 10% SDS, 70µl of 10% APS and 10 µl TEMED

Stacking Gel

Consisted of 3 ml water, .8 ml acrylamide, 1.25 ml Tris Buffer (.5M) pH 6.8, 25µl 10% SDS, 70µl 10% APS and 10µl TEMED.

Tank Buffer

Prepared by dissolving 6.05 gm Tris, 28.8 gm Glycine and 10 ml of 10% SDS to a final volume of 1000 ml using distilled water.

Staining Solution

Prepared by dissolving 0.3 gm coomassie Blue (R-250), 80 ml methanol and 20 ml glacial acetic acid in 100 ml distilled water.

Destaining Solution

Prepared by mixing 100 ml acetic acid, 300 ml methanol and make final volume to 1L using distilled water.

2x Sample Buffer

Prepared by mixing 2.5 ml Tris (0.5 M, pH 6.8), 4 ml SDS (10%), 2 ml glycerol (100%), 0.8 ml β-mercaptoethanol, 300µl bromophenol blue (3%) and 400 µl distilled water.

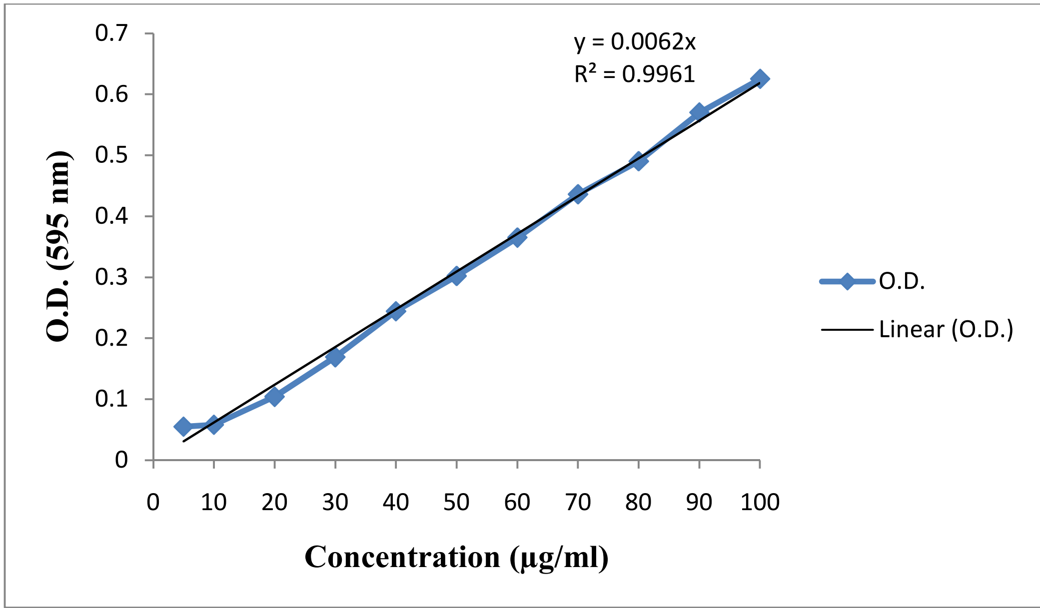
Bradford Assay

Procedure

Different dilutions of BSA were prepared by mixing stock BSA solution and double distilled water. From these dilutions 1 ml protein solution was pippeted out to different test tube and 5ml dye was added to it. Solutions were mixed well and were incubated at room temperature for 30 minutes. Blank was prepared. Optical density was measured at 595 nm. A graph was plotted absorbance against protein concentration. The procedure was repeated for samples and protein concentration was estimated.

Standard Curve of BSA

CONCENTRATION (µg/ml)	ABSORBANCE (595 nm)
Blank	0.000
5	0.055
10	0.058
20	0.104
30	0.121
40	0.203
50	0.302
60	0.336
70	0.436
80	0.490
90	0.510
100	0.625



y = 0.0062x
R^2 = 0.9961
O.D. (595 nm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0 10 20 30 40 50 60 70 80 90 100
Concentration (µg/ml)
O.D.
Linear (O.D.)