Studies On Isolation, Characterization, Antimicrobial Susceptibility And Effect Of Over-the-counter Drugs On Lactobacillus Acidophilus

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Abstract

The gut microflora or the probiotic organisms are the microorganisms inhabiting the digestive tract. There are more than 1000 species residing in various parts of the tract and most of them affect the host beneficially. Lactobacillus species form the predominant group amongst the probiotic organisms. Various sources like yogurt and cheese-whey were used to isolate Lactobacillus Acidophilus. When these were cultured, the most predominant organisms seen were Lactobacillus species and Yeast. Lactobacillus species were selectively isolated and screened further to isolate the Acidophilus. This culture was characterized and maintained in a medium specific to it. A comparative study of the growth kinetic parameters was made in a modified nutrient broth and in Rogosa media. It showed a remarkable difference in growth and was found to propagate profusely in the former media. The population of probiotic organisms in our digestive tract is highly susceptible to antibiotic intake and drug consumption. The extent of inhibitory effects of amoxicillin (ß-lactum), ciprofloxacin (fluoroquinolones) and the Minimum Inhibitory Concentration (MIC) of the two antibiotics were ascertained. Also, the effects of two commonly used Over-The-Counter (OTC) drugs namely a Paracetamol (acetaminophen: commercial name is Dolo-650) and a Non-Steroidal Anti-Inflammatory Drug (NSAID) (Ibuprofen: commercial name is Brufen) were studied. The effect on its viability was more stringent in the case of the antibiotics and in case of the OTC drugs, a minimal reduction in the viability of the culture was observed even at very high concentrations. However, at these concentrations, significant morphological variation was seen and these effects needed further investigation. Index Terms stability, macro-dilution, minimum inhibitory concentration MIC, over-the-counter OTC, susceptibility and tolerance, specific growth, doubling time.

Key words: Antimicrobial Agent, Plant extract, Clinical specimen, Minimum Inhibitory Concentration (MIC)

INTRODUCTION

I. The human body holds a vast internal ecosystem referred to as intestinal microflora, consisting of millions of living microorganisms that coexist harmoniously. Over 400 distinct species of microorganisms inhabit the various

regions of the human digestive tract. If this microflora ecosystem is functioning properly, it guards the human body against harmful bacteria, yeast and virus (Reid, G. et al., 1988). It also stimulates the function of the entire digestive system, produces essential vitamins and regulates their levels. It also maintains proper immune function and counteracts cancer-causing compounds in the colon (Fuller, R., 1994).

Lactobacilli species has recently caught the rapt attention of medical and scientific researchers due to the extraordinary health enhancing benefits they exhibit such as the production of vitamin K. Vitamin K is important in helping blood to clot and is essential for the building of strong bones. *Lactobacillus acidophilus* is the best known of the entire *Lactobacilli* group. This popular resident of the gastrointestinal tract is also widely known for its ability to produce significant quantities of the enzyme lactase, which aids in the digestion of milk and other dairy products, and eliminates many of the serious problems associated with lactose intolerance.

The population balance of the intestinal beneficial microflora is most commonly disrupted by antibiotic usage, excessive sugar consumption, stress and drinking chlorinated water. Researchers have also found that altered levels of acidity and alkalinity in the gastrointestinal tract will change the ecology of the bowel environment, and thereby, affect the type, quantity and behavior of micro organisms found there. Excessive alcohol consumption, frequent use of over-the-counter as well as prescribed anti-inflammatory drugs, painkillers and frequent consumption of carbonated-beverages can also disrupt the population. By considering the potential harmful effects caused by the anti-microbial and OTC drugs towards probiotic population, this study has been proposed to check the effect of the above on *Lactobacilli sp.*

II. MATERIALS

A. Lactobacillus Acidophilus

Lactobacillus acidophilus is Gram-positive bacilli and varies in morphology from long, slender rods to short coccobacilli, which frequently form chains. They are non-motile, catalase negative, fermentative and have specific growth requirements. Some species are aero-tolerant and may utilize oxygen through the enzyme flavoprotein

oxidase, while others are strictly anaerobic. The growth is optimum at pH 5.5-5.8 and the organisms have complex nutritional requirements for amino acids, peptides, nucleotide bases, vitamins, minerals, etc. The isolate was maintained in modified nutrient agar plats (Fig. 1) and slants at 4°C.

B. Amoxicillin

It is a moderate-spectrum, β-lactam antibiotic used to treat bacterial infections. Amoxicillin is susceptible to degradation by β-lactamase-producing bacteria, and so, may be given clavulanic acid to decrease its susceptibility. Amoxicillin acts by inhibiting the synthesis of bacterial cell walls. It inhibits the cross-linkage between the linear peptidoglycan polymer chains that make up the major component of the cell wall of gram-positive bacteria.

C. Ciprofloxacin

It is an antimicrobial drug belonging to a group called fluoroquinolones. Ciprofloxacin is a broad-spectrum antibiotic that is active against both gram-positive and gram-negative bacteria. Its mode of action depends upon blocking bacterial DNA replication by binding itself to an enzyme called DNA gyrase, thereby causing double strand breaks in the bacterial chromosome. It functions by also inhibiting topoisomerase, an enzyme necessary to separate replicated DNA, thereby inhibiting cell division.

D. Paracetamol

It is a common analgesic and antipyretic drug that is used for the relief of fever, headaches, and other minor aches and pains. Paracetamol has long been suspected of having a mechanism of action similar to aspirin because of the resemblance in structure. That is, it has been assumed that paracetamol acts by reducing the production of prostaglandins, which are involved in the pain and fever processes, by inhibiting the cyclooxygenase (COX) enzyme. Without timely treatment, paracetamol overdose can lead to liver failure and mortality associated with paracetamol-induced toxicity.

E. Ibuprofen

It is a non-steroidal anti-inflammatory drug (NSAID). It is used for the relief of symptoms of arthritis, primary dysmenorrhoea, fever, and as an analgesic, especially where there is an inflammatory component. Ibuprofen is believed to work through inhibition of cyclooxygenase (COX), thus inhibiting prostaglandin synthesis. Ibuprofen inhibits both COX-1 and COX-2. It appears that its analgesic, antipyretic, and anti-inflammatory activity are achieved principally through COX-2 inhibition; whereas COX-1 inhibition is responsible for its unwanted effects on platelet aggregation and the gastro intestinal mucosa.

F. Culture Medium

1) Rogosa Agar : This medium is specific for the isolation and enumeration of Lactobacilli sp. Composition (g/liter): Peptone from casein 10.0; Yeast extract 5.0; D(+)glucose 20.0; potassium dihydrogen phosphate 6.0; ammonium citrate 2.0; Tween®80 1.0; sodium acetate 15.0; magnesium sulphate 0.575; iron(II) sulphate 0.034; manganese sulphate 0.12; agar-agar-15.0; the pH was adjusted to 5.5.

2) Modified Nutrient Broth: Composition (g/litre): Yeast Extract-1.5; Beef extract-1.5; Sodium Chloride-5.0; Peptone-5.0; pH was adjusted to 5.5 (De Man, J. C. et al., 1960)

III. METHODS

A. Isolation and Characterization

The microbial source of cheese-whey was collected from primary clarifier tank of M/s. Aavin Dairy & Co-operative firm, Sholinganallur, Chennai and was used to isolate *Lactobacillus acidophilus* (Hilde, M. et al., 2005). Upon culturing, the most predominant organisms seen were *Lactobacillus species* and *Yeast.* From these mixed cultures, *Lactobacillus species* were selectively isolated and screened by comparing their sugar fermentation patterns with the scheme described in Bergey's Manual of Systematic of Bacteriology.

B. Analysis of Microbial Growth

1% inoculum in 200 ml of modified nutrient broth was taken under shake flask cultivation method to study the growth kinetics of *L. acidophilus* and incubated at 35°C. The periodical sterile samplings were done for every 30 minutes with a sample volume of 3 ml. The culture was analyzed for its absorbance at 600 nm with respect to time (Apella, M. C. et al., 1992).

C. Antibiotic Susceptibility

Micro-dilution test was performed to find the Minimum Inhibitory Concentration (MIC) of Amoxicillin, Ciprofloxacin, Paracetamol and Ibuprofen on *L. acidophilus*. The inoculum was allowed to grow up to exponential phase (Sabine Kastner et al., 2006) and test was conducted as per the scheme shown in Table I for antibiotics and Table II for OTC drugs. The experimental cultures were incubated in a shaker at 35°C overnight.

The culture tubes were examined after 18 hours for its growth by absorbance at 600 nm and haemocytometry. The Minimum Inhibitory Concentration value (MIC) was calculated.

TABLE 1. MICRO-DILUTION TEST FOR ANTIBIOTICS

Tube No.	I	II	III	IV	V	VI	VII	VIII	IX	X
Media Volume (ml)	2	2	2	2	2	2	2	2	2	2
Inoculum volume (µl)	200	200	200	200	200	200	200	200	200	200
Conc. Anti - biotic (µg/ml)	2	1	0.5	0.25	0.125	0.062	0.031	0.016	0.00781	0.0039

Microscopic examination of these culture samples was done to determine any morphological changes that may have been induced due to stress by antibiotics.

TABLE 2. MICRO-DILUTION SCHEDULE FOR OTC DRUGS

Tube Number	I	II	III	Control
Volume of media (ml)	2	2	2	2
Inoculum volume (µl)	200	200	200	200
Volume of drug (ml)	2	2	2	-
Conc. of Brufen (mg/ml)	40	4	0.4	-
Conc. of Dolo-650 (mg/ml)	65	6.5	0.65	-

IV. RESULTS AND DISCUSSION

A. *Media Suitability Studies*

The isolated *L. acidophilus* strain was grown in the various commercial media (Table III), so as to ascertain the best-suited media composition.

Rogosa Broth is a selective media which showed high culture purity, but the culture growth was found to be very slow (Table III). Even though other media showed satisfactory results, nutrient broth was selected because of a steady rise in biomass concentration.

TABLE III. COMPARISON OF GROWTH IN VARIOUS CULTURE MEDIA

Time (Hours)	Absorbance at 600 nm			
	Nutrient Broth	Lauria Broth	Rogosa Broth	Peptone Broth
0	0.028	0.033	0.008	0.027
2	0.171	0.139	0.018	0.147
3	0.412	0.360	0.075	0.210
4	0.807	0.437	0.100	0.384
6	1.320	0.516	0.293	0.416

Fig.1 Modified Nutrient Agar-L. Acidophilus

B. *Analysis of Microbial Growth*

The kinetic parameters were checked for the isolate (LA 2) and compared with control strain *Lactobacillus acidophilus* MTCC-2459 (LA 1).

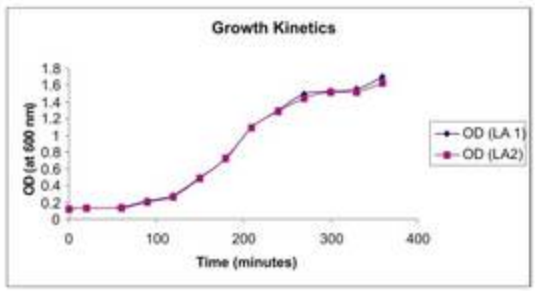

Fig. 2 A plot of ln(OD) Vs. Time

LA 1(Control): Specific growth rate, µ: 0.01214 (min) and Doubling Time (T_d): 57.09 (min)

LA 2 (Isolate): Specific growth rate, µ: 0.01160 (min) and Doubling Time (T_d): 59.75 (min)

C. *Effect of Glucose on Growth of Lactobacillus Acidophilus (LA 2)*

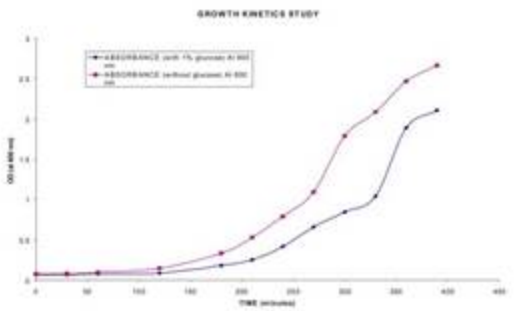

Fig. 3 a plot of OD vs. Time

Concentration of Brufen (mg/ml)	Absorbance (at 440 nm)
0	2.261
40	2.206
4	2.064
0.4	0.743

Concentration of Dolo-650 (mg/ml)	Absorbance (at 600 nm)
0	2.258
0.65	2.140
6.5	1.889
65	1.599

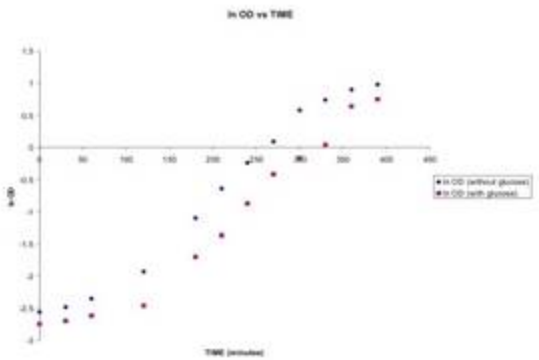

Fig. 4 a plot of ln (OD) Vs. Time

Amoxicillin concentration (µg/ml)	Absorbance (at 600 nm)
0	1.766
0.03125	0.005
0.04	0.005
0.0475	0.004
0.055	0
0.0625	0

Without glucose: Specific growth rate, µ: 0.01160 (min) and Doubling Time (T_d): 59.75 (min)

With glucose : Specific growth rate, µ: 0.0139 (min) and Doubling Time (T_d): 49.72 (min)

The presence of excess glucose did not increase the growth rate of the culture but an increased lag time could be seen (Fig. 3).

D. Antibiotic Susceptibility

The minimum inhibitory concentrations (MIC) for the antibiotics were found as mentioned below:

TABLE IV. MINIMUM INHIBITORY ASSAY FOR AMOXICILLIN

It is found that the MIC of amoxicillin against Lactobacillus acidophilus was 0.055 (µg/ml).

For Ciprofloxacin, the micro-dilution tests revealed that *L. acidophilus* was susceptible between the concentrations 0.25 µg/ml and 0.125 µg/ml. It was noticed that further increase in concentration makes the culture grow as aggregates. Thus, MIC was determined using haemocytometry.

Ciprofloxacin of concentration 0.2 g/ml on isolate showed 18.2×10^{5} CFU/ml whereas, the control test showed 73×10^{6} CFU/ml. There had been a reduction in the growth of 97%. Thus, the MIC of ciprofloxacin against *Lactobacillus acidophilus* was determined as 0.2 µg/ml.

TABLE V. MINIMUM INHIBITORY ASSAY FOR IBUPROFEN

E. Tolerance to Over-The-Counter Drugs

The minimum inhibitory assay tests for ibuprofen (Table V) and a paracetamol (Table VI) showed growth at all concentrations. However, the absorbance showed a reduction in growth with increasing concentrations of the drug.

F. Morphological Variations

As per the microscopic observation, the morphology of the cells in the presence of antibiotics and OTC drugs changed considerably. The rods seemed elongated and two to three cells joined end-to-end to form short chains. This effect seemed more pronounced with increase in antibiotic and OTC drug concentrations.

V. CONCLUSION

Lactobacillus acidophilus (LA2) was isolated and characterized from the cheese-whey sample. Its susceptibility and tolerance were tested against commonly used OTC drugs and antibiotics. There was no remarkable decrease (Tables IV and V) in the growth in

the presence of OTC drugs. However, there were drastic morphological changes like the clumping of cells and the elongation of rods that are induced due to stress by drug concentration.

Drugs are now becoming a necessary aspect for survival with the increasing incidences of various illnesses. Many of the human pathogens are fast becoming resistant to the antibiotics that are being used to treat these diseases and so, the concentration of the prescribed antibiotics/drugs is being increased. All these aspects adversely affect the growth and survival of the gut microflora.

There are a few possibilities by which the ill effects on gut microflora can be reduced such as supplementation of lyophilized organisms (Salzmann, S. et al., 1996) to replenish the biomass content and introduction of genetically engineered probiotic organisms with antibiotics resistant genes. It is significant that the engineered genes should be made non-transferable to the pathogens. Further scope of study is to identify the tolerance of these organisms by mimicking the body conditions with respect to the pH, temperature, bile salts and bioavailability of the drugs.

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