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RESEARCH ARTICLE

Synthesis of Bacteriocin by Synbiotic Effect and Its Antibacterial Activity against Selected Respiratory tract Pathogens

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Abstract

Probiotics produce essential protein peptides in the form of bacteriocin that have an antimicrobial effect in controlling the harmful pathogenic bacteria. These extra cellular peptides are bacteriocins that have a bactericidal mode of action on related pathogenic bacterial species. Probiotics and its secondary metabolites are the Nutraceutical supplements for many kinds of living system and indirectly it helps in the process of eliminating the intruding pathogens. The current work ensured that the probiotic enzyme activity is highly enhanced along with the selective prebiotics (synbiotic enzymatic action) against the selected respiratory tract pathogens (*Streptococcus pneumoniae and Staphylococcus aureus*). The zone of inhibition produced by the prebiotic (ie., *Punica granatum*) extract impregnated discs showed higher zone of control against *S. pneumoniae* (17mm) and against *S. aureus* (23mm) respectively. These prebiotic modes of actions were compared with selective antibiotics like Erythromycin (23mm/17mm) and Amoxicillin (19mm/16mm) for *S. pneumoniae and S. aureus* respectively. So, this present study conferred an overview of bacteriocin synthesis by probiotics in the presence of selective prebiotics (synbiotic effect) and its antibacterial activity against disease causing pathogens (selected respiratory tract pathogens).

Introduction

Upper respiratory tract (nose, sinus, pharynx, and larynx) infections are caused by nasal tract pathogens like *Staphylococcus aureus* and *Streptococcus pneumoniae.* Many antibiotics are in the market to prevent or reduce the illness caused by upper respiratory tract pathogens but the risk of using antibiotics is that induces the pathogen to become the antibiotic resistant bacteria. But, most of the time it does not discriminate between the beneficial and harmful bacteria.

Recent studies were being evidenced in that the probiotic strains can prevent respiratory infections**.** The work (Kaarina K et al., 2008) supposed that infants fed with the synbiotics promotes maturation of the immune system, which results in a 13 % risk reduction for respiratory infections from 6 to 24 months of age of newly born infants.

. According to Fuller (1989) the probiotics are defined as "probiotics are a living microbial feed supplement which beneficially affects the host by improving its intestinal microbial balance". Many researchers proved the efficacy of probiotics and its applications in various fields (aquaculture, formulating the animal feed, treatment of various gastrointestinal tract diseases, etc).

Many bacterial infections are treated with natural compounds which possess Prebiotics elements. The definition for prebiotics has since been revised as 'a selectively fermented ingredient that allows specific changes,

both in the composition and/or activity in the gastrointestinal microflora that confer benefits upon the host well-being and health' (Gibson *et al.,* 2004). Prebiotics have been suggested to have several beneficial effects, including promotion of beneficial bacterial growth, production of short chain fatty acids, and a shortened orofecal transit time (Cummings *et al.,* 1997). Prebiotics are not digested by the enzymes secreted by the human intestine as they are having a low calorific value and are efficiently used in low calorie foods. In this perspective, prebiotics are efficiently used to increase the rate of recurrence and to treat constipation (Kleessen *et al.,* 1997, Den Hond *et al.,* 2000). Along with the suitable prebiotics, the probiotics may improve a person's health by regulating their immune function; it is called as '*Synbiotics'*.

A *synbiotic* is an additive that contains both prebiotics and probiotics. Any supplement that contains both pre- and probiotics, those two works in association to maintain the system with good and beneficial bacteria in sufficient quantities. The probiotic survival and the gut bacterial activities are increased by the presence of prebiotics in the form of synbiotic mixture (Underwood MA *et al*., 2009, Vlieger AM *et al.,* 2009, Chouraqui JP *et al*., 2008, Puccio G *et al.,* 2007). No relevance research work has examined the impact of synbiotics on clinical outcomes against pathogenic forms and its formulations in fed term infants. But recent systematic reviews of research groups (published from 2007 to 2011) on the use of prebiotics or probiotics in term infants have focused on prevention of allergic disease and food hypersensitivity (Kleessen *et al.,* 1997, Den Hond *et al.,* 2000). In children and adults, upper respiratory tract infections, antibiotic associated diarrhea and acute infectious diarrhea were reviewed by Qiukui H *et al.,* (2011) and it focused on full term eradication of respiratory tract pathogens with the supplementation of synbiotics (i.e. probiotics and prebiotics). Synbiotics have been characterized as healthy food with prebiotics and probiotics properties improving the human health (Gibson GR *et al.,* 1995). Production of synbiotic foods have been increasingly focusing on health benefits by imparting resistance to infectious diseases causing pathogens, antibacterial activity, and improved immunity (Gibson GR *et al.,* 1995).

Bacteriocins are antibacterial proteins produced by bacteria that kill or inhibit the growth of other bacteria. Many lactic acid bacteria produce a high diversity of different bacteriocins. Bacteriocins are ribosomally synthesized peptides or proteins with antimicrobial activity produced by many gram-positive and gram-negative bacteria. Moreover the bacteriocin characteristic features can be studied based on its biosynthesis process and its genetic origin. Basic understanding of the structure–function, biosynthesis, and mode of action and other many aspects of these compounds (bacteriocin) are still unknown. Some of Lactic acid bacteria (LAB) produce bacteriocins, antibacterial proteinaceous substances with bactericidal activity against related species (narrow spectrum) or across genera (broad spectrum of activity). Maximal bacteriocin production could be obtained by supplementing a culture medium with growth limiting factors, (like sugars, vitamins and nitrogen sources and by regulating pH) or by suitable culture medium (Vignolo et al., 1995). Bacteriocin biosynthesis is a desirable characteristic for strain selection as it serves as an important mechanism of pathogen exclusion in fermented foods as well as in the gastrointestinal environment. LAB bacteriocins are small antimicrobial peptides or proteins that possess activity towards closely related most of the Gram-positive bacteria may cause inhibitory action by their own synthesized bacteriocins. Lactic acid bacteria bacteriocins are generally stable in acid or neutral pH, indicating that the substances are well adapted to the environment of the bacteria producing them.

This present study is mainly focused on the bacteriocin synthesis and its antimicrobial activity by the probiotics (*Lactobacillus rhamnosus* & *Lactobacillus plantarum*) incorporated with selected prebiotics against the respiratory tract pathogens, i.e. *Staphylococcus aureus* and *Streptococcus pneumoniae*.

Materials and Methods

Probiotic Organisms

The probiotics, *Lactobacillus rhamnosus, Lactobacillus plantarum* were obtained from IMTECH, Chandigarh. Bacterial strains were sub – cultured for 24 hours at 35° C in 5% CO_2 and 95% air atmosphere and maintained in laboratory condition for future observation.

Pathogenic Strains

The pathogens *Staphylococcus aureus* and *Streptococcus pneumoniae* were obtained from the patient of PSG Institute of medical science and hospital, Coimbatore cultured in MRS agar, Chocolate agar medium and then they were incubated at 35°C and 5% CO2 for 48-72 hours and maintained under the same conditions.

Selection of Prebiotics

The Prebiotic samples were chosen according to the presence of oligosaccharides, sugar and fibers. The chosen prebiotic sources are *Prunus dulcis, Zea mays, Borassus flabellifer, Aloe vera, Jersaleum artichoke, Punica granatum, Cichorium intybus, Asparagus, Allium sativa, Allium cepa.*

Antibacterial activity of Prebiotics by Disc diffusion method

The antibacterial activities of the selected ten Prebiotics were assessed by the disc diffusion method against the selected respiratory tract pathogens *Staphylococcus aureus,* and *Streptococcus pneumonia.* They were compared with the standard antibiotics (Erythromycin and Amoxicillin).

The test organisms were *Staphylococcus aureus*, and *Streptococcus pneumoniae*. They were cultured in nutrient broth for a period of 24 hours. The MRS agar and Chocolate agar were prepared and sterilized by autoclaving at 121°C for 15mins. Then the media were allowed to cool up to palm bearable temperature, and then the media were poured in Petri plates and allowed to solidify. Using a sterile cotton swab, the potential pathogenic cultures (*Staphylococcus aureus* and *Streptococcus pneumoniae*) were spread on the agar plates. After swabbing, the plates were placed with disc soaked in prebiotics extract (100µl). The standard antibiotic discs were also placed in the agar plate and sterile disc is also maintained as a control. Then the plates were incubated at 35°C for 24 hours. After incubation, the plates were assessed for the antimicrobial activity by the formation of the zone of inhibition around the disc. By measuring the diameter of the zone, the antimicrobial activity of each prebiotic extract was compared with the antibiotic treatments.

Determination of enzymatic activity of bacteriocin

Enzymatic activity of bacteriocin produced by the probiotic bacteria *Lactobacillus plantarum* and *Lactobacillus rhamnosus* cultured with the prebiotic medium were assessed by well diffusion method against the selected respiratory tract pathogens.

Evaluation of bacteriocin activity

Bacteriocin activity was determined for *Lactobacillus plantarum* and *Lactobacillus rhamnosus,* in the presence of various prebiotic incorporated media by agar-well diffusion method. MRS agar plates were prepared and pathogenic strain *Streptococcus pneumoniae* was swabbed. The wells were made under sterile condition in the MRS agar plates with selected pathogen lawn culture.

To find the measure of enzyme activity and synthesis of bacteriocin, 10^7 cells of probiotic culture were made. The probiotic culture *Lactobacillus plantarum* and *Lactobacillus rhamnosus* were grown overnight for 24 hours. The potential probiotic cultures *L.plantarum* and *L. Rhamnosus* were washed with freshly prepared and sterile MRS broth by centrifugation at 4000 rpm for 10 minutes. The pellet was suspended in MRS broth and 4% of probiotic suspension was added in nine different prebiotics incorporated and Control (without addition of any prebiotics) MRS broth flasks.

After incubation, the cultures were centrifuged at 10000 rpm for 20 minutes and the collected supernatant was serially diluted up to 10^{-3}µl. The serially diluted supernatant (60 µl) were added into the wells of previously prepared pathogen lawn cultured plates. Similarly, serially diluted probiotic supernatant were poured into the well for every two hours interval and each of the plates were incubated at 33°C for 24 hours and observed in the enzymatic inhibitory spectrum against selected pathogens. The clear zone of inhibition around the each well indicates the presence of bacteriocin produced by probiotics.

Dynamics of Bacteriocin production

The enzymatic activity of bacteriocin produced by the probiotics with the combination of prebiotics (*Lactobacillus rhamnosus, Lactobacillus plantarum, Lactobacillus plantarum* + prebiotic, and *Lactobacillus rhamnosus* + prebiotic) were read by arbitrary units based on the zone of inhibition around the each well. Arbitrary unit was defined as the reciprocal of the highest dilution yielding zone of inhibition in the indicator lawn (Mayr-Harting *et al.,* 1972). The arbitrary unit values were found to be higher than 3%.

Results

Antimicrobial activity of prebiotics against pathogens

The respected medium for *Staphylococcus aureus* and *Streptococcus pneumoniae* were used to test the antagonistic effect of the plant extract (prebiotics) impregnated discs. They were compared with the standard

antibiotics, to find out whether the prebiotic compound possesses antibacterial properties or not. Out of ten prebiotic plant extracts, seven of them showed a promising result in antagonistic tests (Table 1). Therefore seven prebiotics were selected for checking the antibacterial property and compared with antibiotics. *Punica granatum* incorporated disc showed higher zone of control (23mm radius) when compared to 17mm and 16mm by Erythromycin and Amoxicillin respectively. The other plant extracts also have antimicrobial property; zone of control is lesser than that of antibiotic disc in the case of *Staphylococcus aureus* cultured MRS plates (Table 1). In the case of *Streptococcus pneumonia*, antimicrobial discs showed larger zone of control (erythromycin-23mm and Amoxicillin-17mm), followed by *Punica granatum*-17mm, *Borassus flabellifer*-10mm and *Aloe vera*-9mm.

Test for enzymatic activity

In agar well diffusion method used for estimation of bacteriocin activity, Lactobacillus *plantarum* with 10^{-1}, 10^{-2}, and10^{-3} dilutions at respective two hours incubation showed high zone of control with various prebiotics, whereas the *Lactobacillus plantarum* alone without any prebiotics incorporated showed less zone of control due to very slow growth of probiotics and lack of prebiotics (Table 2). The bacteriocins are produced largely in the presence of prebiotics *Prunus dulcis, Borassus flabellifer, Jersaleum artichoke, Asparagus* at the end of ten hour incubation. In the case of *Punica granatum,* enzymes were synthesized at an earlier stage and then gradually the enzymatic activity decreased. Probiotic (Control) without any prebiotic supplementation showed very slow growth and lesser enzyme activity in arbitrary units. This research report thus proves that prebiotics are the essential supplement of food required for probiotics to grow rapidly.

In agar well diffusion method used for estimation of bacteriocin activity, *Lactobacillus rhamnosus* at 10^{-1}, 10^{-2}, and10^{-3} dilutions with two hours incubation showed high zone of control with various prebiotics, whereas the *Lactobacillus rhamnosus* alone without any prebiotics incorporated showed less zone of control due to very slow growth of probiotics and lack of prebiotics (Table 3). The bacteriocins are produced largely in the presence of prebiotics *Prunus dulcis, Borassus flabellifer, Jersaleum artichoke, Asparagus* at the end of ten hour incubation in the presence of *Lactobacillus plantarum*. Probiotics (Control) without any prebiotic supplementation showed very slow growth and lesser enzyme activity in arbitrary units.

Bacteriocin synthesis

The bacteriocin is an enzyme that causes an inhibitory spectrum on the pathogenic organism. The bacteriocin production is mainly estimated on the basis of growth phase. Bacteriocin production is examined in different prebiotic and probiotic combination and probiotic alone i.e., (*Lactobacillus rhamnosus, Lactobacillus plantarum, Lactobacillus plantarum* + prebiotic, and *Lactobacillus rhamnosus* + prebiotic). The rate of bacteriocin production reached height after 8 hours in synbiotic supernatant inoculated, whereas the Control (probiotic) alone promoted the synthesis of bacteriocin very slowly. Increase in bacteriocin production is detected by the inhibition zone formed on the pathogenic organism cultured in the plates.

Table 1. Inhibitory response of prebiotic incorporated sterile disc and antibiotic disc against pathogens

S.NO	PREBIOTICS	ZONE OF INHIBITION (cm)	
		Staphylococcus aureus	***Streptococcus pneumonia***
1	*Aloe vera*	0.8	0.9
2	*Punica granatum*	2.3	1.7
3	*Cichorium intybus*	0.9	0.5
4	*Allium cepa*	0.5	0.5
5	*Borassus flabellifer*	0.8	0.8
6	*Asparagus*	0.6	0.5

7	*Allium sativa*	0.5	0.4
8	Erythromycin	1.7	2.3
9	Amoxycillin	1.6	1.9

Table 2. Bacteriocin effect of *Lactobacillus plantarum* (probiotic) with various prebiotics against *Streptococcus pneumoniae*

S.No	**PREBIOTICS**	**ARBITRARY UNITS IN TIME INTERVALS(AU/ml)**				
		2nd hour	**4th hour**	**6th hour**	**8th hour**	**10th hour**
1.	*Prunus dulcis*	0.05	0.05882	0.07	0.0833	0.078
2.	*Zea mays*	0.01	0.02	0.04	0.04545	0.037
3.	*Borassus flabellifer*	0.043	0.06	0.065	0.076	0.064
4.	*Aloe vera*	0.033	0.0365	0.04	0.045	0.04
5.	*Jersaleum artichoke*	0.1	0.1	0.09	0.0833	0.08
6.	*Punica granatum*	0.0833	0.04545	0.04545	0.033	0.025
7.	*Cichorium intybus*	0.04	0.0833	0.09	0.125	0.10
8.	*Asparagus*	0.033	0.05	0.055	0.066	0.064
9.	*Allium sativa*	0.033	0.035	0.0357	0.04	0.035
10.	Control	0.012	0.02	0.032	0.04	0.045

*Control: Probiotic- *Lactobacillus plantarum* (with no prebiotics added)

Table 3. Bacteriocin effect of *Lactobacillus rhamnosus* (probiotic) with various prebiotics against *Streptococcus pneumoniae*

S.No	**PREBIOTIC SOURCE**	**ARBITRARY UNITS IN TIME INTERVALS (AU/ml)**				
		2nd hour	**4th hour**	**6th hour**	**8th hour**	**10th hour**
1.	*Prunus dulcis*	-	0.028	0.07	0.077	0.076

2.	*Zea mays*	-	-	0.04	0.058	0.032
3.	*Borassus flabellifer*	-	0.02	0.065	0.069	0.060
4.	*Aloe vera*	-	0.03	0.04	0.044	0.037
5.	*Jersaleum artichoke*	-	0.09	0.09	0.093	0.062
6.	*Punica granatum*	-	0.044	0.045	0.047	0.021
7.	*Cichorium intybus*	-	0.049	0.09	0.13	0.089
8.	*Asparagus*	-	0.02	0.055	0.065	0.061
9.	*Allium sativa*	-	0.023	0.035	0.037	0.030
10.	Control(only probiotic)	-	0.019	0.022	0.042	0.042

*Control: Probiotic- *Lactobacillus rhamnosus* (with no prebiotics added)

Discussion

Results of antibacterial activity of prebiotic plant extracts (Table 1) against both pathogens are evidence to prove that the prebiotic compound from plant extracts are having some degree of antimicrobial capacity. This might be supported by previous research work (Jehan A. & S. Salman, 2009), the antimicrobial activity of chicory extract had given 12 mm inhibitory zone against pathogenic *E. coli* and concluded that the effect of antibacterial activity this may be due to the contents presents in the chicory content like a dietary fiber and oligosaccharides.

The outcome of the current research reports was primarily focused on determining cultural conditions for obtaining better and stable bacteriocin production. *L. plantarum* along with a supplement of prebiotics was able to synthesize bacteriocin, which posses a wide inhibitory spectrum towards both pathogenic bacteria (*Staphylococcus aureus* and *Streptococcus pneumoniae*). It inhibited the indicator strains with the largest zone of inhibition. The bacteriocin produced by *L.plantarum* alone had a lesser inhibitory effect on the indicator strains (Table 1).

This was concluded with the support of previous work (Jehan A. & S. Salman, 2009); the combined effect (synergistic effect) of pre- and probiotics (synbiotics *Bifidobacterium sp.* + chicory and Bifidobacterium *sp.* + inulin) on pathogenic *E. coli* were higher when compared to the effect of Bifidobacterium *sp.* alone and chicory alone.

Bacteriocin production by the probiotic produced primary metabolite kinetics. i.e., bacteriocin was synthesized in large amount during the pre-and early exponential growth phases and reached a maximum level at early stationary phase. But, some reports propose that bacteriocins are produced throughout the growth phase and not only during late logarithmic or early stationary phase (Joerger and Klaenhammer, 1986). Results prove that bacteriocin was produced when nutrients were utilized for metabolism. Thus, higher amount of the bacteriocin was synthesized only when the medium was supplemented with *Prunus dulcis, Zea mays, Borassus flabellifer, Aloe vera, Jersaleum artichoke, Cichorium intybus, Asparagus, Allium sativa.* Thus when diverse prebiotic supplementation is provided for the cultivation of probiotic, the amount of bacteriocin produced by microorganisms is higher. An older report specifies that a modification of nutrients of cultivation media should be considered for higher production of bacteriocin which has high potential as a food preservative (Biswas et al., 1991).

Finally concluded with this, the bacteriocin was synthesized by selected probiotics in the presence of suitable plant based prebiotics which acts as the essential substrate for the probiotics. This biosynthesized bacteriocin acts upon the pathogenic protein which was produced by the disease causing pathogens. Hence to conclude the synbiotics (prebiotic and probiotic) complexes has been attributed to its excellent outcome in affording

with antimicrobial effect and enzymatic activity against the selected respiratory tract pathogens (*Staphylococcus aureus* and *Streptococcus pneumoniae*). Additional in – vivo studies are warranted to identify synbiotic effects on infections caused by the respiratory tract pathogens and their immunological mechanisms.

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