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Synergistic Interaction of Probiotics in Multispecies Filtrate Form against Food Borne Pathogens

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ABSTRACT: Previous studies suggested that the multi-strain probiotic preparation exhibits better inhibitory activity against food borne pathogens as compared to single strain probiotic preparation. Thus, in the present study, two different probiotic bacteria were prepared as multi-species filtrate form and evaluated for their antibacterial properties against selected four food borne pathogens. *Lacticaseibacillus casei* and *Bifidobacterium bifidum* was selected as probiotics in the present study. Against all the pathogens, multispecies probiotic filtrate showed high inhibition capacity. The recorded zone of inhibition was found greater with multispecies probiotic filtrate as compared to mono-strain form. In calculation of GIIs, the interaction was found synergistic between the selected probiotics. Thus, probiotics in mixed form might be useful for treatment of food borne diseases.

Keywords: *Bifidobacterium bifidum*, Food borne pathogens, *Lacticaseibacillus casei*, Multispecies, Mono-strain, Probiotic Filtrate, Synergistic effect.

INTRODUCTION

Probiotics are used for a long time as food ingredients for humans and also used for animals to feed them without any side effects. Also, probiotics are acceptable because of being naturally present in the intestinal tract of healthy humans and foods (Çakir, 2003). The history of probiotics goes way back to the history of humans when fermented foods were consumed. The term probiotic comes from the Greek language "Probios" which means 'for life'. This term is opposite to the term "antibiotics" which means 'against life'. Originally in 1953, Kollath suggested the term probiotic and denoted all organic and inorganic food complexes as probiotics in contrast to harmful antibiotics. The purpose of such practice was to upgrade these food complexes as supplements. Technically the term probiotics are defined by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO), as "live microorganisms which upon ingestion in certain numbers exert health benefits beyond inherent general nutrition" (Joint FAO/WHO, 2001 and 2002). In other words, probiotics are defined as live microorganisms that positively affect the host's health when administered in appropriate amounts (Brown and Valiere 2004; Kalliomaki *et al.,* 2001). There are different forms of probiotic preparations such as mono-strain, multi-strain or multispecies probiotics, and each probiotic form shows different efficacy (Aalaei *et al.*, 2018). A mono-strain probiotic is defined as containing one strain of a certain species while the term multispecies probiotics are used for preparations containing strains that belong to one or preferentially more genera. It is important that the strains used in multi-strain and multispecies probiotics preparation should be compatible or, preferably, synergistic. In an earlier study, Timmerman *et al.* (2004) found multispecies probiotics preparation remarkable in the treatment of AAD in children, clearance of *E. coli* O157:H7 from lambs and inhibition of *S. Enteritidis* in rats as compared to mono-strain and multi-strain probiotic preparations. Vidyalaxme *et al.* (2014) reported the synergistic effect of multispecies probiotic preparation against *Vibrio cholera* in ragi malt. Astolfi *et al.* (2019) also reported detoxification of heavy metals in nursing mothers and their infants when the mother consumed multi-strain probiotic preparation.

In view of the above, this experiment was designated to evaluate the synergistic interaction between *Lacticaseibacillus casei* and *Bifidobacterium bifidum* in multi-species probiotic filtrate form. The present work is a continuation of our previous work in which we studied *Lacticaseibacillus casei* and *Bifidobacterium bifidum* against four pathogens in mono-strain form (Raisagar and Shukla 2022a). Now in the present work, our aim is to find out the interaction between selected probiotic bacteria in multi-species filtrate form and to evaluate the effectiveness of the multi-species filtrate preparation against the four pathogens in comparison with mono-strain probiotic form.

MATERIAL AND METHODOLOGY

Place of the work. All the in vitro experiments were performed in the Dairy Food Quality and Safety Laboratory, Department of Dairy Microbiology, WCDT, SHUATS, Prayagraj, U.P. India.

Selection of food-borne pathogens. *Escherichia coli, Staphylococcus aureus, Shigella dysenteriae* and *Salmonella typhi* were selected as food-borne pathogens in the present study. The selected food borne pathogens were isolated and identified in previous study (Raisagar *et al.*, 2022).

Probiotic strains procurement. Two probiotic strains namely *Lacticaseibacillus casei* and *Bifidobacterium bifidum* were procured from the National Collection of Industrial Microorganisms (NCIM), Pune in dried culture form. The dried cultures were revivedby followingstandard procedure of revival.Probiotic potentials of selected strains were confirmed in previous study (Raisagar and Shukla 2022b).

Preparation of multi-species probiotic filtrate. For the preparation of multi-speciesprobiotic filtrate, both selected probiotics were inoculated separately in 10ml of MRS broth and incubated for 18 hours at 37°C. After incubation, probiotic broth cultures containing 10^8 CFU/ml were separately centrifuged at 6000 rpm for 15 to 20 min to remove cells. Then the supernatants were collected and sterilized by filtration with a 0.2 µm pore-size filter and referred as filtrate. Each probiotic filtrate was mixed in equal amount to prepare multi-species probiotic filtrate (Bayoumi and Griffiths 2012). Prepared multi-species probiotic filtrate was stored at 4°C until further use (Yu *et al.*, 2013; Pehrson *et al.*, 2015).

Antibacterial activity of mixed probiotic filtrates against isolates. The antibacterial activity of multi-species probiotic filtrate against food borne pathogens was studied by using the agar well diffusion method (Abdel-Raouf *et al.*, 2014). Muller Hinton agar was pour plated with a single indicator strain of the food borne pathogen. A 6 mm well was bored in the MHA plate with help of a sterile cork borer and filled with the multi-species probiotic filtrate.The plate was incubated at 37°C for 24 hours. After that incubation inhibition zone was recorded.

Determination of interaction. To determine the interaction between selected probiotic bacteria, the growth inhibitory indices (GIIs) were calculated using the formula suggested by Mandal *et al.* (2010). The formula was given below:

$$GIIs = \frac{\text{Clear zone obtained by probiotic bacteria in mutispecies filtrate form}}{\text{Total of clear zones of both probiotics in monostrain form}}$$

The synergistic and antagonistic interaction between the selected probiotic bacteria in multi-species probiotic filtrate form was defined with GIIs > 0.5 and GIIs < 0.5, respectively.

RESULTS AND DISCUSSIONS

In the present study, antibacterial activity of probiotic cultures against isolated food borne pathogens (*Escherichia coli, Staphylococcus aureus, Shigella dysenteriae* and *Salmonella typhi*) were evaluated in combination form as multi-species probiotic filtrate. Multi-species probiotic filtrate of *Lacticaseibacillus casei* and *Bifidobacterium bifidum* showed antibacterial activity against all aimed food borne pathogens. Earlier several researchers also reported the antibacterial activity of probiotic filtrate against human pathogens in their studies (Saud *et al.*, 2020, Abdelhamid *et al.*, 2018; Chowdhury and Islam 2016; Gad *et al.*, 2016); Halimi and Mirsalehian 2016; Chakraborty and Bhowal 2015; Nigam *et al.*, 2012). Antibacterial activity of *Lactobacillus casei* filtrate against multidrug-resistant *Shigella sonnei* and *Shigella flexneri* was studied by Mirnejad *et al.* (2013) whereas Mirzaei *et al.* (2018) studied the inhibition of *Shigella* species by cell free supernatant of *Lactobacillus paracasei* and *Lactobacillus rahmnosus*. Similarly, Masalam *et al.* (2018) reported antibacterial activity of cell free supernatant of *Lactobacillus* against food borne pathogens *Escherichia coli, Staphylococcus aureus, Salmonella* and *Shigella* species whereas Huang *et al.* (2015) reported inhibition of these pathogens by cell free supernatant of *Lactobacillus plantarum*. In the present study, the highest zone of inhibition was recorded against pathogen *Escherichia* coli whereas the lowest zone of inhibition was against pathogen *Shigella dysenteriae*. The highest zone of inhibition (ZOI) recorded for muti-species probiotic filtrate against *Escherichia coli* was 40 mm, followed by *Salmonella typhi* (ZOI = 35 mm) and *Staphylococcus aureus* (ZOI = 32 mm) whereas the lowest ZOI was recorded against *Shigella dysenteriae* with 30 mm ZOI (Table 1). The results are in agreement with research conducted by El-Jakee *et al.* (2010) in which multi-species filtrate preparation of three probiotics namely *Lactobacillus acidophilus, Bifidobacterium bifidum* and *Streptococcus thermophilus* inhibits the growth of *Salmonella* species with ZOI of 10 mm. Antibacterial activity of multi-species probiotic filtrate was also reported by Hamad *et al.* (2017) where multi-species filtrate of three probiotics namely *Bifidobacterium bifidum, Lactobacillus acidophilus* and *Lactobacillus plantarum* showed inhibition against growth of *Streptococcus pyogenes* and *Escherichia coli* O157:H7. The study also showed that the recorded ZOI was greater in the case of multi-species probiotic filtrate as compared to mono-strain probiotic filtrate preparation (Fig. 1).

Table 1: Antibacterial activity of multi-species probiotic filtrates against pathogens.

Isolates	Zone of Inhibition (mm)
Escherichia coli	40
Staphylococcus aureus	32
Shigella dysenteriae	30
Salmonella typhi	35

Data are average of three replications

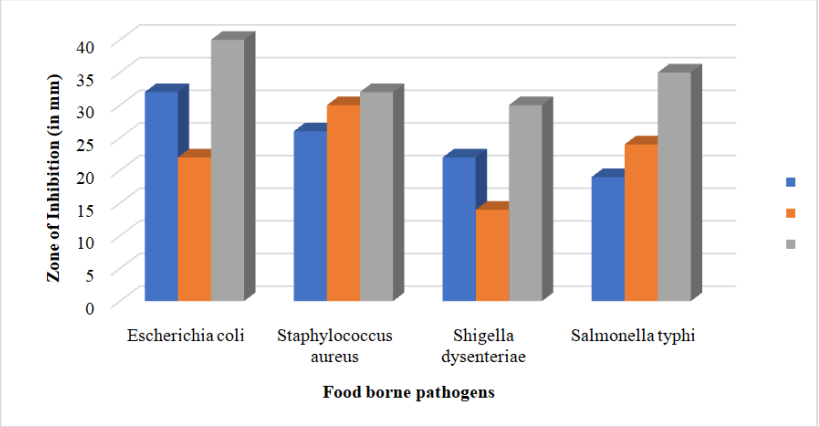

Fig. 1. Comparison between antibacterial efficacy of mono-strain and multi-species probiotic filtrate.

In the present study, the width of clear zone (R) was also calculated using the formula suggested by Carasi *et al.* (2014); Pisano *et al.* (2014). The calculated R for multi-species probiotic filtrate was 17 mm against *Escherichia coli*, 14.5 mm against *Salmonella typhi*, 13 mm against *Staphylococcus aureus* and 12 mm against *Shigella dysenteriae*. Inhibition scores were considered as the no inhibition capacity with R < 2 mm; low inhibition capacity when R = 2 to 5 mm, and high inhibition capacity with R > 6 mm (Table 2).

Table 2: Width of clear zone (R) and inhibition capacity of multi-species probiotic filtrate.

Isolates	Width of clear zone (R), mm	Inhibition Capacity
Escherichia coli	17	High
Staphylococcus aureus	13	High
Shigella dysenteriae	12	High
Salmonella typhi	14.5	High

R < 2 mm= no inhibition capacity; *R* = 2 to 5 mm =low inhibition capacity; R > 6 =high inhibition capacity

In the present study, the growth inhibitory indices (GIIs) were calculated using the formula to determine the interaction between selected probiotic bacteria. The interaction can be synergistic or can be antagonistic when two or more probiotics were in multi-species form. If GIIs were more than 0.5 the interaction was considered synergistic and the interaction was antagonistic when GIIs were less than 0.5. The calculated GIIs against all the tested pathogens were greater than 0.5. GIIs for multi-species probiotic filtrate against *Escherichia coli* was 0.71, 0.57 for *Staphylococcus aureus*, 0.54 for *Shigella dysenteriae* and 0.63 for *Salmonella typhi*. The calculated GIIs showed that the selected probiotic bacteria had synergistic interaction when used as multi-species probiotic filtrate forms (Table 3).

Previously, Chen *et al.* (2018) recorded the synergistic inhibitory effect of probiotic supernatant *Lactobacillus fermentum* and bovine lactoferrin against multidrug resistant *Staphylococcus aureus*. Tian *et al.* (2010) also reported an increment in antibacterial activity against pathogens by use of cell free supernatant of *Lactobacillus reuteri* in combination with bovine lactoferrin. To prevent and control food borne diseases or infections in humans, Uraipan and Hongpattarakere (2015) suggested the use of probiotic products developed by the combination of *Lactobacillus* and *Bifidobacterium*. In the present study, multi-species filtrate of selected probiotic bacteria showed antibacterial effects against tested pathogens. A similar observation was also made by Chakraborty and Bhowal (2015 with cell free supernatant of *Lactobacillus*. The study suggested the cause of such behavior was the production of inhibitory metabolites which were extracellular and diffusible. This was also supported by Patra *et al.* (2011).

Table 3: GIIs and type of interaction between the probiotic cultures in mixed filtrate form.

Isolates	Growth inhibitory indices (GIIs)	Type of interaction
Escherichia coli	0.71	Synergistic
Staphylococcus aureus	0.57	Synergistic
Shigella dysenteriae	0.54	Synergistic
Salmonella typhi	0.63	Synergistic

GIIs > 0.5 Synergistic interaction and GIIs < 0.5 Antagonistic interaction

CONCLUSIONS

In the present study, the selected probiotic bacteria *Lacticaseibacillus casei* and *Bifidobacterium bifidum* possess antibacterial activity against selected food borne pathogens and showed synergistic interaction in combined form as multi-species probiotic filtrate. Multi-species probiotic filtrate showed higher zone of inhibition against pathogens as compared to mono-strain form. Thus, the study also concluded that to get a better result in food borne disease prevention, probiotic bacteria would be used as multi-species filtrate form. However, further study is required to check the

efficiency of selected probiotic culture in multi-species filtrate form against another enteric and food borne pathogens. There is also a need of in vivo study of effect of multi-species probiotic filtrate against tested food borne pathogens.

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Conflict of Interests. None.

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