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PERILLA FRUTESCENS EXTRACT TARGETING PREBIOTIC ACTIVITY

IN VITRO STUDY TO EXPLORE PREBIOTIC EFFECTS ON PROBIOTIC AND PATHOGENIC BACTERIA

KEYWORDS: Perilla frutescens, digestive health, prebiotic, microbiota, irritable bowel syndrome, Benegut, microbiome, Peer Reviewed.

ABSTRACT

Perilla frutescens (L.) extract is used in food supplements to support the immune system and GI comfort. A proprietary **Perilla frutescens** leaf extract was used to investigate prebiotic effects on strains of Lactobacillus, Bifidobacterium and pathogenic bacteria by testing for growth stimulation effects on agar plates. Perilla frutescens leaf extract was confirmed to be a prebiotic, supporting the growth of selected lactobacilli and bifidobacteria and inhibiting the growth of pathogenic bacteria. Results underline beneficial effects in the application in blends with probiotics preventing inflammations, supporting the immune system and to improve intestinal health.

INTRODUCTION

The microbiota of the human intestine is a balanced system, which consists of a vast assemblage of microorganisms, which ensure the structural integrity of the gut mucosal barrier, immune function, and protection against pathogens. Prebiotic substances are selectively digested by intestinal bacteria, like *Lactobacillus* or *Bifidobacterium*, to support their growth. At the same time, prebiotics limit the growth of pathogenic strains, like for example Enterobacteria (1). Furthermore, prebiotics are metabolized by intestinal strains, potentially leading to the production of short chain fatty acids like acetate or butyrate. These are important to maintain a healthy gut motility and to protect against intestinal inflammation (2).

Traditionally, non-digestible carbohydrates, oligosaccharides, are the main source of prebiotics substances in the human diet, for example fructo-oligosaccharides (FOS) (1). Their effective dosage is several grams, which is not convenient for food supplement applications, where the quantity for a capsules or tablet is limited. Furthermore, bloating is a common side effect, which make these types of prebiotics not suitable for all consumers. Therefore, an increasing interest is noted for alternative prebiotics, based on plant flavonoids, which are effective at lower dosages and provide additional benefits being antioxidants and anti-inflammatory compounds. In the presented study, a proprietary *Perilla frutescens* leaf extract (PE), standardized on flavonoids, was investigated for prebiotic effects. *Perilla frutescens* (L.) Britton is an annual edible herbaceous plant native to Asia. Common names are Shiso or Japanese Basil. It is a member of the family Lamiaceae. Perilla leaves are used as tea, food or spice (3, 4). The proprietary PE was used to investigate potential prebiotic activity on the growth of probiotic and pathogenic bacteria by carrying out growth experiments on agar plates with and without additional C- source.

MATERIALS AND METHODS

Characterization of Perilla leaf special extract

PE was obtained by water extraction of dried *Perilla frutescens* (L.) leaves. PE contains a specific ratio of selected flavonoids, particularly vicenin 2, which has been investigated for gut health (5–9). The extract is commercially available under the brand name Benegut® and is a proprietary ingredient of Vital Solutions GmbH, manufactured by Amino Up Co., Ltd. In this study native PE in test solutions of 1% was investigated.

Figure 1. *Perilla frutescens field.*

Selection of probiotic and non-probiotic strains

The growth behavior of ten probiotic strains and eight non-probiotic strains was tested on agar plates. Table 1 shows the selected strains. Probiotic strains were selected based on common application for GI health. Pathogenic strains were selected based on the risk for infection during travelling.

The study was conducted by Organo Balance GmbH, now Novozymes Berlin GmbH.

MEDIA

Media for Lactobacillus strains

LMM medium (Lactobacillus minimal medium):

di-potassium hydrogen phosphate 2 g/l, di-ammonium hydrogen citrate 2 g/l, calcium chloride dehydrate 0.5 g/l, magnesium sulphate heptahydrate 0.6 g/l, guanine 0.1 g/l, cytosine 0.1 g/l, thymidine 0.1 g/l, 2´-desoxyuridine 0.1 g/l, 2´-desoxyadenosine 0.1 g/l, cyanocobalamine 0.02 g/l, riboflavin 10 mg/l, folic acid 0.2 g/l, pyridoxal-5-phosphate monohydrate 10 mg/l, aminobenzoate 0.2 g/l, D (+)-biotin 1 mg/l, ascorbic acid 500 mg/l, nicotinacid 10 mg/l, calcium panthotenate 10 mg/l, thiamine 1 mg/l, cobalt-(II)-nitrate hexahydrate 0.5 g/l, manganese (II) sulphate 0.02 g/l, Na_2MoO_4 0.04 g/l, trypticase peptone 15 g/l, tween 80 1 g/l, D-glucose 20 g/l

sMRS medium (synthetic de Man, Rogosa, Sharpe):

Lactobacillus sensu lato Strains	*Bifidobacterium* Strains	Pathogenic Stains
L. acidophilus	*B. bifidum*	*Bacteroides fragilis*
L. bulgaricus	*B. animalis* subspecies *lactis*	*Clostridioides difficile*
L. casei	*B. longum* subspecies *infantis*	*Clostridium perfringens*
L. fermentum	*B. longum* subspecies *longum*	*Enterobacter cloacae*
L. plantarum		*Escherichia coli*
L. rhamnosus		*Klebsiella pneumoniae*
		Proteus vulgaris
		Salmonella typhimurium

Table 1. Selected lactobacilli and bifidobacteria as well as pathogenic strains.

proteose peptone No. 3 10 g/l, beef extract 10 g/l, yeast extract 5 g/l, diammonium hydrogen-citrate ($(NH_4)_2$H-Citrate) 2 g/l, tween 80 1 g/l, magnesium sulphate ($MgSO_4$ x 7 H_2O) 206 mg/l, manganese sulphate ($MnSO_4$ x H_2O) 56 mg/l, di-potassium hydrogen phosphate (K_2HPO_4) 2 g/l, D(+)-glucose x H_2O 20 g/l

Media for Bifidobacterium strains

LMM medium supplemented with (0.5 g/l) cysteine

BM medium (special Bifidobacterium media):

proteose peptone No. 3 10 g/l, beef extract 5 g/l, yeast extract 5 g/l, di-potassium hydrogen phosphate (K_2HPO_4) 3 g/l, sodium ascorbate 1 g/l, L-cysteine 0.5 g/l

Media for Enterobacteria

Enterobacteriaceae CASO medium (facultative anaerobic):

casein peptone 17 g/l, BBL phytone peptone papaic digest 3 g/l, sodium chloride 5 g/l, K_2HPO_4 2 g/l, D(+)-glucose monohydrate 20 g/l (for positive control)

Media for obligate anaerobic bacteria

M110 medium (used for Clostridia difficile, Clostridia perfringens and Bacteroides fragilis):

meat extract 17 g/l, casitone 30 g/l, yeast extract 5 g/l, K_2HPO_4 5 g/l, D(+)-glucose-monohydrate 20 g/l (for positive control), resazurine 25 mg/l, L-cystein 0.5 g/l

Growth experiments set up for lactobacilli and bifidabacteria

On the agar plates, first the test solutions, positive control (1% and 2% glucose), or negative control (C-source free) were added followed by the corresponding media.

The initial pH of the media was adjusted to neutral pH value to stimulate GI conditions. Neither *Perilla frutescens* nor glucose did change the pH value of the tested media. For the spotting on agar plates different dilutions of each of the probiotic strains, starting concentration 10^9 CFU/ml, dilution factors 10^{-1} to 10^{-4}, were prepared. After spotting 5 µl of different dilutions of the bacteria, the inoculated agar plates were incubated at 37 °C under anaerobic conditions for 72 h. The growth of the probiotic strains on the agar plates with the different C-sources was evaluated by photo documentation after 24 h, 48 h, 72 h, and 96 h. Each experiment was done in duplicate.

Growth experiments set up for pathogenic strains

On the agar plates, first the test solutions, positive control (1% and 2% glucose), or negative control (C-source free) were added followed by the corresponding media. After the preparation of agar plates, different dilutions of each of the non-probiotic strains,starting concentration 10^9 CFU/ ml, dilution factors 10^{-3} to 10^{-6}, were prepared and 5 µl spotted onto the agar plates. The inoculated agar plates were incubated at 37 °C under anaerobic conditions. The growth of the non-probiotic strains on the agar plates with the different C-sources was visually evaluated by photo documentation in suitable time intervals dependent on the strain. enterobacteria after 16 h and 24 h incubation, clostridia strains after 24 h and 48 h incubation and *Bacteroides fragilis* only after 7 days of incubation.

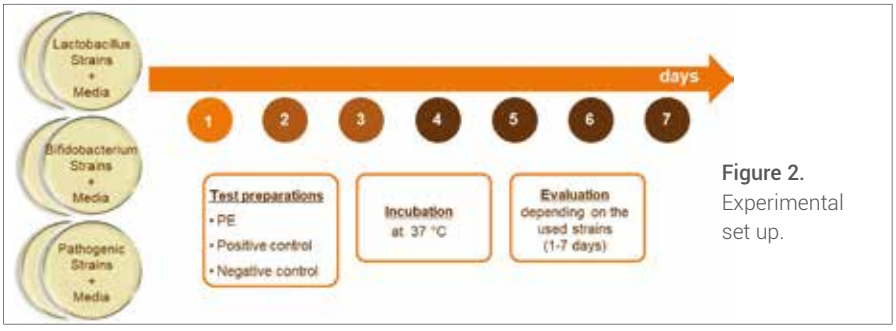

Figure 2. Experimental set up.

The strength of the effects of the lactobacilli and bifidobacteria were classified in two categories. One plus indicating superior growth and two plusses suggesting strong superior growth.

Effect on pathogenic strains were classified in four categories. One minus indicating no stimulation of growth, two minuses inhibition of growth, three minuses complete inhibition of growth and one plus suggesting no inhibition of growth.

RESULTS

PE effect on probiotic strains

As expected, all strains did show growth on the positive controls and no or very limited growth on the negative control. The most obvious results for these strains were obtained after 48 hours of incubation. Therefore, results at this time point are presented. PE showed strong prebiotic effects for all selected probiotic strains and in both growth media. Exceptional growth was demonstrated for *L. acidophilus, L. bulgaricus, B. bifidum*, and *B. longum* subspecies *longum*. The following table summarized the results.

Strain	1 % PE	
	Media	
Bifidobacterium	BM	LMMc
B. bifidum	++	++
B. lactis	+	+
B. longum subspecies *infantis*	+	+
B. longum subspecies *longum*	++	++
	Media	
Lactobacillus sensu lato	sMRS	LMM
L. acidophilus	++	++
L. bulgaricus	++	++
L. casei	+	+
L. fermentum	+	+
L. plantarum	+	+
L. rhamnosus	+	+

Table 2. Effects on probiotic strains.

In the following part the detailed results are given by photo documentation. In the first column the investigated strains are listed. The second and third columns show results for the positive controls and the fourth for the negative control. The last column presents the results for PE on bacterial growth. All tests were done with 4 different dilutions of the bacterial strains,starting concentration 10^9 CFU/ml, dilution factors 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} (from left to right). All selected commensal strains developed superior growth after PE supplementation, comparable to glucose as C-source and *L. acidophilus, L. bulgaricus*, and *B. bifidum* showed strong superior growth (Figure 3, 4). *B. longum* subspecies *longum* was tested separately as it requires a longer cultivation time period. This strain indicated also strong superior growth (Figure 5).

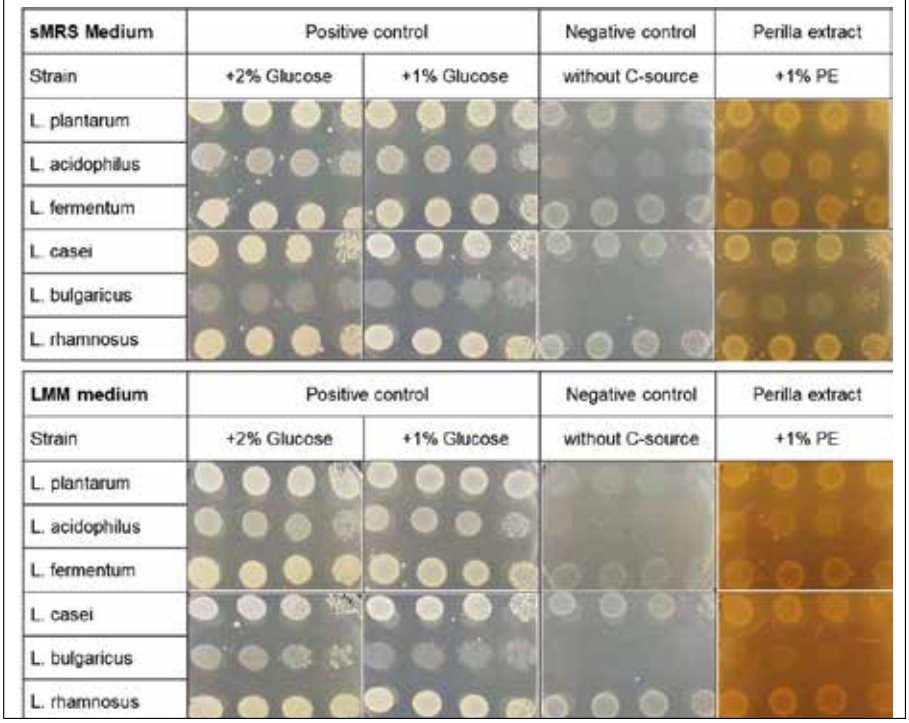

Figure 3. Growth of supplemented lactobacilli on sMRS and LMM media after 48 h cultivation.

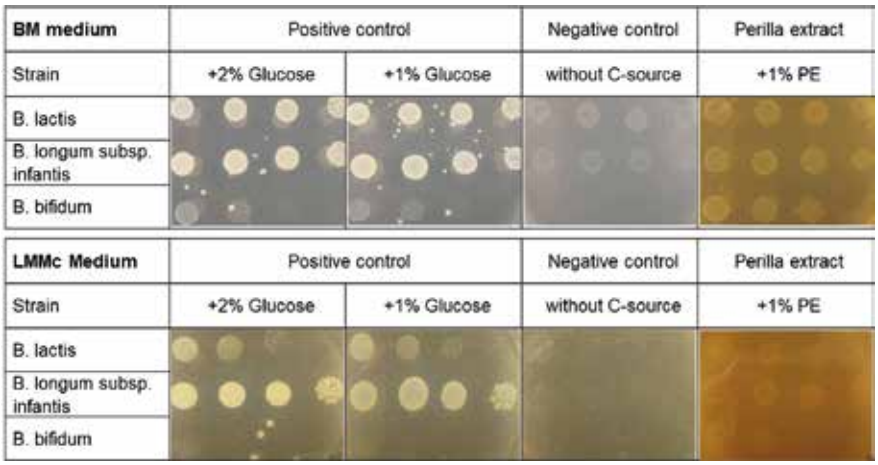

Figure 4. Growth of bifidobacteria on supplemented BM and LMMc media after 48 h cultivation.

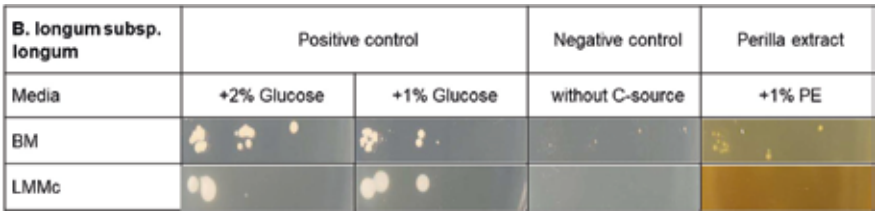

Figure 5. Growth of bifidobacteria subsp. longum on supplemented BM and LMMc media after 96 h cultivation.

PE effect on non-probiotic strains

All strains did show growth on the positive controls and limited growth on the negative control. Results for Enterobacteria are given after 24 h incubation, results for Clostridia strains correspond to 48 h incubation and the results for Bacteroides fragilis are related to 7 days incubation.

Prebiotics are substances which support the growth of probiotic strains and do not support the growth of pathogenic strains. Not supporting growth means, no growth stimulation higher than the positive control, a reduction, or a complete inhibition of growth. PE demonstrated a reduction of the growth of Escherichia coli and Enterobacteria cloacae. The development of Salmonella typhimurium was completely inhibited. Klebsiella pneumoniae and Bacteroides fragilis did not show superior growth compared to positive control. PE could not inhibit the growth of Proteus vulgaris, Clostridioides difficile, and Clostridium perfringens. The following table summarized the study results.

In the following part the detailed results are given by photo documentation. In the first column the investigated strains are listed. The second and third columns show results for the positive controls and the fourth for the negative control. The last column presents the results for PE on bacterial growth. All tests were done with 4 different dilutions of the bacterial strains, starting concentration 10^9 CFU/ml, dilution factors10^{-3}, 10^{-4}, 10^{-5} and 10^{-6},from left to right). PE confirmed prebiotic effects for *Escherichia coli, Enterobacter cloacae*, and *Salmonella typhimurium*, showing a superior inhibition of growth compared to the positive controls (Figure 6). For Klebsiella pneumoniae and Bacteroides fragilis also prebiotic effects were identified as no stimulation of growth compared to the C-sources of the positive controls was seen (Figure 6). PE showed no prebiotic effects on *Proteus vulgaris, Clostridioides difficile*, and *Clostridium perfringens* (Figure 6).

DISCUSSION

The experiments demonstrated prebiotic effects of PE for selected probiotic strains by supporting their growth and for selected pathogenic by inhibiting their development, under in vitro conditions. *L. acidophilus, L. bulgaricus, B. bifidum* and *B. longum* subsp. *longum* showed superior growth after PE supplementation compared to the positive control, glucose, which is usually considered as the optimal C-source for microorganisms.

Interestingly, these probiotic strains were investigated for the reduction of GI discomfort, irritable bowel syndrome (IBS), and to support the immune system (10, 11) similar to PE extract. In a randomized, placebo-controlled, double-blind human pilot study, this PE significantly improved gastrointestinal discomfort symptoms (5). Overall, the mode of action seems to be a combination of antispasmodic as well as anti-inflammatory effects, leading to an immediate, perceptible relief of GI discomfort and a balanced gut motility (6-9).

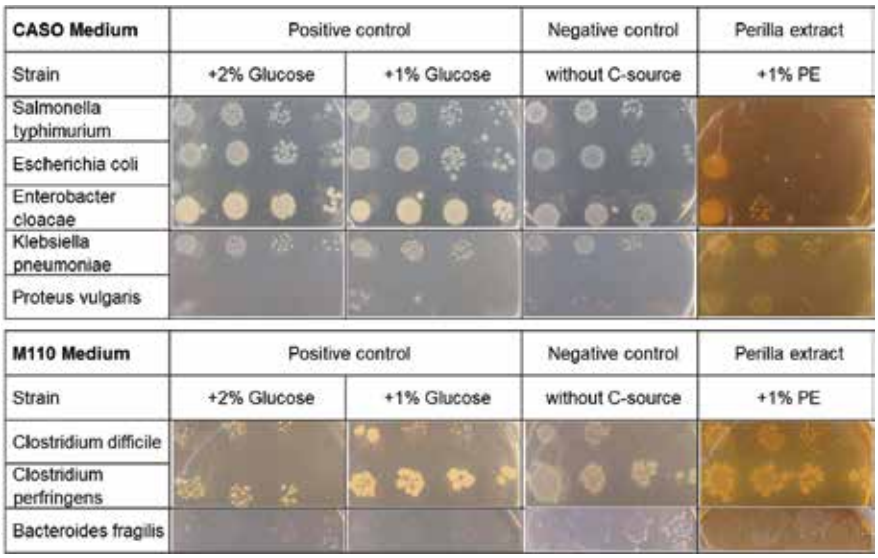

Figure 6. Results on supplemented CASO medium (24 h of cultivation), on M110 medium (Clostridia strains - 48 h of cultivation and Bacteroides fragilis - 7 days cultivation).

Strain	1 % PE
	Media
Enterobacteria	Enterobacteriaceae CASO
Enterobacter cloacae	--
Escherichia coli	--
Klebsiella pneumoniae	-
Proteus vulgaris	+
Salmonella typhimurium	---
	Media
Obligate anaerobic bacteria	M110
Bacteroides fragilis	-
Clostridium difficile	+
Clostridium perfringens	+

Furthermore, PE inhibited the growth of the pathogenic bacteria, like *Escherichia coli, Enterobacter cloacae,* and *Salmonella typhimurium*. Moreover, *Klebsiella pneumoniae* and *Bacteroides fragilis* growth was not stimulated by PE. These beneficial effects to reduce pathogenic strains, could open new applications of PE for travel GI discomfort and diarrhoea (12, 13). To our knowledge, not many plant extracts have been studied for prebiotic effects, however, first studies were published for blueberry extract and grape extract, showing prebiotic effects for Lactobacillus and Bifidobacterium strains (14, 15).

Even less studies are published about plant extracts inhibiting the growth of pathogenic strains. Though, it is reported that Cacao flavanols stimulate growth and proliferation of *Bifidobacterium* spp. and *Lactobacillus sensu lato* spp. and inhibit clostridia growth (16). PE extract is a promising innovative prebiotic and an interesting alternative to fibres. Due to its small dosage and its low water activity, it can be incorporated in dietary supplements in combination with probiotics or used as stand-alone ingredient. It could even be used synergistically together with fibre targeting prebiotic effects as well as to support GI discomfort based on its soothing and bowel balancing benefits. Our current data can be considered as an initial proof of concept, providing first results for the prebiotic effects of PE. To confirm our data, further in depths investigations are required to confirm effects in humans.

CONCLUSION

PE was confirmed to have prebiotic potential, supporting the growth of probiotic bacteria and inhibiting the growth of pathogenic bacteria. Results underline its beneficial effects for digestive health and the potential application to avoid travel GI discomfort related to travel infections.

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