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Growth Responses of Lactic Acid Bacteria to Leguminous Seed Extracts

Hoi-Seon Lee and Young-Joon Ahn*

Division of Applied Biology & Chemistry and the Research Center for New Bio-Materials in Agriculture, College of Agriculture and Life Sciences, Seoul National University, Suwon 441-744, Korea

Abstract : Methanol extracts from 25 seed samples belonging to the family Leguminosae were subjected to an *in vitro* screening for their growth-promoting and inhibitory activities towards *Bifidobacterium adolescentis, B. longum, B. bifidum,* and *Lactobacillus casei,* using spectrophotometric and paper disc agar diffusion methods under O_2-free conditions, respectively. The responses varied with both bacterial strains and plant species. Among seed extracts, extracts from *Glycine max* (light-green color) and *Arachis hypogaea* (dark-brown) enhanced the growth of lactic acid bacteria in media with or without carbon sources, suggesting that bifidus factor(s) might be involved in the phenomenon. This growth-promoting effect was most pronounced with *L. casei* among lactic acid bacteria used. Additionally, all seed extracts did not adversely affect the growth of the lactic acid bacteria tested.(Received January 24, 1997; accepted February 17, 1997)

Introduction

Approximately 400 kinds of microorganisms are resident in the human intestinal tract as a highly complex ecosystem with considerable species diversity. The intestinal microbiota in healthy subject remains relatively constant but is known to be greatly influenced by physical, biological, chemical, environmental or host factors.[1-3] They not only participate in normal physiological functions, but also contribute significantly to the genesis of various disease states by biotransforming a variety of ingested or endogenously formed compounds to useful or harmful derivatives. These biotransformations may influence drug efficacy, toxicity, carcinogenesis and aging.

Previous investigations have demonstrated that there were some differences in intestinal bacteria between patients and healthy control subjects,[4,5] and between the younger and elderly subjects.[2,6,7] The normal gastrointestinal microbiota is found to be predominantly composed of lactic acid bacteria which seem to play an important role in metabolism, host defense against infection, aging and immunopotentiation.[1,2,8] On the other hand, the microbiota of cancer patients is composed of a high concentration of clostridia and eubacteria with few lactic acid bacteria. It has also been reported that elderly subjects harbour fewer bifidobacteria but larger numbers of clostridia than younger subjects. Accordingly, any disturbance of the microbiota may cause a variety of diseases of abnormal physiological states.

In relation to human health, much current concern has focused on plant-derived bifidus factors which promote the growth of beneficial bacteria and plant-derived growth inhibitors against harmful bacteria such as *Clostridium perfringens* and *Escherichia coli* because plants constitute a rich source of bioactive chemicals.[9] However, relatively little work has been carried out on the effect of leguminous seed extracts on growth of intestinal microorganisms compared to other areas of intestinal microbiology in spite of their excellent nutritional and industrial significance.[10,11]

In the laboratory study described herein, we assessed the growth-promoting and inhibitory responses of lactic acid bacteria to extracts of 25 leguminous seeds.

Materials and Methods

Bacterial strains and culture conditions

The intestinal bacterial strains used in this study were as follows; *Bifidobacterium bifidum* ATCC 29521, *B. longum* ATCC 15707, *B. adolescentis* ATCC 15073, and *Lactobacillus casei* ATCC 14916. Stock cultures of four strains were routinely stored on Eggerth-Gagnon Liver Extract-Fieldes slant[12] at -60℃ and were subcultured on Eggerth-Gagnon agar[12] (EIKEN Chemical, Tokyo, Japan) when required, and were incubated anaerobically at 37℃ for 2 days in a Glove box (Coy Co., USA) in an atmosphere of 5 per cent H_2 + 15 per cent CO_2 + 80 per cent N_2.

Key words : lactic acid bacteria, leguminosae, growth promotion, growth inhibition
*Corresponding author

Plant materials and sample preparation

The leguminous seeds purchased as a commercially available product were dried in a oven at 60℃ for 3 days, finely powdered, extracted twice with methanol at room temperature and filtered (Toyo filter paper No. 2). The combined filtrate was concentrated *in vacuo* at 35℃. The yield of each plant extraction is shown in Table 1. The nutritional and industrial importance of leguminous seeds are provided by Sharpe,[10] and Smith and Huyser.[11]

Microbiological assay

For growth measurements with microorganisms, the testing methods of Mitsuoka[13] and Ahn *et al.*[14] were applied. In the experiments for bifidus factor(s) derived from non-carbon sources, György[15] broth (pH 6.8) as modified by Yoshioka[16] was used. In the experiments for bifidus factor(s) derived from carbon sources, MRS[17] broth (pH 7.8) was used. Ahn *et al.*[18] already reported that 10~20 mg/disc of a plant extract did not cause any problems such as insolubility and difficulty in detection of its minor active components. One per cent of each seed was inoculated onto the media described above, and 10 mg of each membrane filter-sterilized test material was added to the media in a final volume of 10 m*l*. Solutions of the test materials were prepared using methanol as solvent. The methanol concentration in the solutions did not exceed 2 per cent which was found to be without adverse effect on the bacteria tested. The media were incubated anaerobically at 37℃ for 2 days, and the bacterial growth spectrophotometically measured at 600 nm. Growth-promoting response was expressed as growth increase rate (GIR=A_{600} sample/A_{600} reference). The responses were determined as previously described: strong response +++, GIR>2.0; moderate ++, 2.0>GIR>1.6; weak, 1.5>GIR>1.0; and no response -, GIR<1.0. Each assay was replicated three times.[17]

For assay of the inhibititory effect of the test samples on the organisms, one loopful of bacteria was suspended in 1 m*l* of sterile physiological saline. An aliquot (0.1 m*l*) of the bacterial suspensions was seeded on EG agar. A sample (10 mg) dissolved in methanol was applied by Drummond glass microcapillary to a paper disc (ADVANTEC 8 mm Toyo Roshi, Japan). After evaporation, the paper discs were placed on EG agar surface. They were incubated at 37℃ for 2 days in an atmosphere of 5 per cent H_2 + 15 per cent CO_2 + 80 per cent N_2. Control discs received methanol. All tests of inhibition were replicated three times. The growth responses of test samples were determined by comparison with those of controls. The inhibitory responses were classified as previously described: strong response +++, zone diameter>20 mm; moderate ++, zone diameter 16~20 mm; weak +, zone diameter 10~15 mm; no response ·, and zone diameter <10 mm.[18]

Table 1. List of leguminous plants tested

Scientific name	Color (abbreviation)	Korean name	Yield[a] (%)
Amphicarpaea edgeworthii var. *trisperma*	Purple (P)	새콩	10.7
Arachis hypogaea	Dark-brown (Dbr)	땅콩	5.3
Canavalia lineata	Brown (Br)	해녀콩	12.0
Cassia obtusifolia	Dark-brown (Dbr)	결명자	13.3
Dunbaria villosa	Light-brown (Lbr)	새돔부	5.6
Glycine max	Black(B_1-size: 1.1 cm)	서리태	10.0
Glycine max	Black(B_2-size: 0.5 cm)	약콩	5.5
Glycine max	Black(B_3-size: 0.8 cm)	흑태	6.6
Glycine max	Dark-brown (Dbr)	방콩	5.4
Glycine max	Dark-purple (Dp)	금두	4.8
Glycine max	Light-green (Lg)	청태	11.1
Glycine max	Purple (P)	울타리콩	1.9
Glycine max	Yellow (Y)	메주콩	7.1
Glycine soja	Brown (Br)	돌콩	10.7
Lathyrus japonica	Black (B)	갯완두	12.0
Phaseolus multiflorus	Dark-purple (Dp)	붉은 강낭콩	5.3
Phaseolus nipponensis	Dark-green (Dg)	새팥	5.7
Phaseolus radiatus	Black (B)	거두	7.8
Phaseolus radiatus	Green (G)	녹두	5.2
Pisum sativum	Light-green (Lg)	완두	3.6
Rhynchosia volubilis	Brown (Br)	넝쿨들콩	5.3
Vicia hirsuta	Black (B)	새완두	11.8
Vicia tetrasperma	Light-purple (Lp)	얼치기 완두	12.3
Vigna angulasis	Red (R)	적두	4.8
Vigna sinensis	Light-yellow (Ly)	동부	6.2

[a](weight/dried weight of sample)×100.

Results and Discussion

Growth-promoting activity of extracts from 25 leguminous seed samples towards lactic acid bacteria used was investigated. For determination of bacterial growth, two kinds of media were used: modified György broth as a carbon source-containing medium and MRS broth as a carbon source-free medium. The growth responses varied with plant species and bacteria tested.

Growth-promoting activity of the test bacteria to extracts from the seed samples in modified György broth is shown in Table 2. In tests with *B. adolescentis* and *B. longum* which are predominant in the intestines of adults, extracts from all samples exhibited weak (+) or no promoting activity. With *B. bifidum* which is dominant bacteria in the intestines of infants, weak or no growth-promoting activity was obtained in all samples. For *L. casei*, extracts from *G. max* (light-green color), *G. max* (black), *R. volubilis* (green), and *V. angularis* (red) strongly enhanced the growth of this bacteria (+++), whereas moderate growth-promoting responses

Table 2. Effect of leguminous seed extracts on the growth of lactic acid bacteria in modified György broth

Sample (color)[a]	Strain			
	B. adolescentis	*B. longum*	*B. bifidum*	*L. casei*
A. edgeworthii var. *trisperma* (P)	-	-	+	+
A. hypogaea (Dbr)	-	+	+	-
C. lineata (Br)	-	+	-	-
C. obtusifolia (Dbr)	-	+	+	+
D. villosa (Lbr)	-	+	+	++
G. max (B_1)	-	+	-	++
G. max (B_2)	-	-	-	+++
G. max (B3)	-	+	+	+++
G. max (Db)	-	+	-	+
G. max (Dp)	-	+	+	++
G. max (Lg)	-	+	+	+++
G. max (P)	-	-	-	++
G. max (Y)	-	+	+	++
G. soja (Br)	-	-	+	+
L. japonica (B)	-	+	+	+
P. multiflorus (Dp)	+	+	+	+
P. nipponensis (Dg)	-	+	-	+
P. radiatus (B)	-	+	-	+
P. radiatus (G)	-	+	-	++
P. sativum (Lg)	-	-	-	-
R. volubilis (Br)	-	+	+	+++
V. hirsuta (B)	-	+	+	-
V. tetrasperma (Lp)	-	+	-	+
V. angulasis (R)	-	-	+	++
V. sinensis (Ly)	-	+	+	+

[a]For explanation, see Table 1.

Table 3. Effect of leguminous seed extracts on the growth of lactic acid bacteria in MRS broth

Sample (Color)[a]	Strain			
	B. adolescentis	*B. longum*	*B. bifidum*	*L. casei*
A. edgeworthii var. *trisperma* (P)	-	+	+	+
A. hypogaea (Dbr)	+	++	++	+
C. lineata (Br)	+	-	+	-
C. obtusifolia (Dbr)	+	+	+	+
D. villosa (Lbr)	-	+	-	+
G. max (B_1)	-	+	+	+
G. max (B_2)	-	-	-	-
G. max (B_3)	-	+	+	+
G. max (Dbr)	-	-	+	+
G. max (Dp)	-	-	-	+
G. max (Lg)	++	++	++	+
G. max (P)	-	-	-	-
G. max (Y)	+	+	+	+
G. soja (Br)	-	-	-	+
L. japonica (B)	-	+	+	+
P. multiflorus (Dp)	+	+	+	+
P. nipponensis (Dg)	-	+	-	+
P. radiatus (B)	-	-	+	+
P. radiatus (G)	-	-	+	+
P. sativum (Lg)	+	+	-	-
R. volubilis (Br)	-	+	+	+
V. hirsuta (B)	-	+	-	+
V. tetrasperma (Lp)	+	-	-	-
V. angulasis (R)	-	-	-	-
V. sinensis (Ly)	-	+	++	+

[a]For explanation, see Table 1.

(++) were produced from 8 samples. The promoting effect was most pronounced with *L. casei* among lactic acid bacteria used.

Table 3 shows growth-promoting activity of lactic acid bacteria to 25 seed samples in MRS broth. In tests with *B. adolescentis, G. max* (light-green) exhibited moderate growth-promoting activity. Growth of *B. longum* and *B. bifidum* was moderately affected by the addition of extracts from *G. max* (green color) and *A. hypogaea* (dark-brown). However, weak or no promoting activity against *L. casei* was obtained from all seed samples.

The growth inhibition of four bacterial strains to 25 leguminous seed samples was investigated using paper disc agar diffusion method (data are not shown). All samples did not adversely affect growth of these lactic acid bacteria tested.

Among the various human intestinal microorganisms, bifidobacteria are often taken as useful indicators of human health under most environmental conditions. This is based upon the facts that they play important roles in metabolism, *e.g.* nutrition production such as vitimin and essential amino acid, aid defense against infection, are assocoated longevity, pathogen inhibition, immunity activation, improvement of lactose tolerance of milk products, and antitumorigenic activity.[1,3,19,20] Bifidobacteria growth-promoting factors, usually called bifidus factor, have therefore been extensively studied since György *et al.*[21] suggested their existence in human milk. Bifidus factors are classified into lacteal secretions, fructooligosaccharides, derivatives of lactose, and xylooligosaccharides.[22]

In our microbial study, extracts from *G. max* (light-green) and *A. hypogaea* (dark-brown) enhanced the growth of lactic acid bacteria in media with or without carbon sources, suggesting that bifidus factor(s) might be involved in the phenomenon. It would be most desirable to both inhibit the growth of potential pathogens and/or increase the numbers of bifidobacteria in the human gut. Selective growth promoters for bifidobacteria or inhibitors for harmful bacteria are especially important for human health, because intake of these materials may normalise disturbed physiological functions which result in the prevention of diseases caused by pathogens in the gastrointestinal tract. Similar *in vitro* results were also reported in extracts from ginseng,[14] soybean,[23,24] and green tea.[25,26] Recent *in vivo* investigations using human volunteers have shown that intake of ginseng extract,[27] green tea-derived polyphenols,[28] and soybean-derived insoluble oligosaccharides containing stachyose or raffinose[29] affected favorably faecal microbiota and biochemical aspects of faeces, suggesting an indication of at least one of their pharmacological actions. Accordingly, the daily intake of leguminous seed-derived materials might be expected to alter the growth and composition of the microbial community and modulate the genesis of potentially harmful products such as carcinogenic *N*-nitroso compounds of aromatic steroids within the intestinal tract, thus protecting from a variety of diseases and maintaining optimal human health.

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콩과식물 종실 추출물의 유산균에 대한 생육반응

이회선, 안용준*(서울대학교 농업생명과학대학 농생물학과 및 농업생물신소재연구센터)

초록 : 26종 콩과식물 종실의 메탄올 조추출물의 *Bifidobacterium adolescentis, B. longum, B. bifidum, Lactobacillus casei*에 대한 생육촉진 및 생육억제 활성을 탄소원 배지와 비탄소원 배지를 이용하여 *in vitro* 혐기조건하에서 각각 분광학적 및 여지확산법으로 검정한 결과 생육반응은 공시세균과 콩의 종류에 따라 달리 나타났다. 콩과식물 종실중, 대두(밝은 청색 종실)와 땅콩 (짙은 갈색 종실) 추출물은 유산균의 생육을 촉진하였는데, 이러한 결과로 부터 이들 콩과식물 종실에 탄소원과 비탄소원의 비피더스 인자의 존재를 알 수 있었다. 공시 유산균중에서 *L. casei*에서 가장 현저한 생육촉진 반응을 나타내었다. 또한, 모든 콩과식물 종실시료는 상기 유산균의 생육을 저해하지 않았다.

찾는말 : 유산균, leguminosae, 생장 촉진, 생장 저해

*연락저자