Anti-Pathogenic Activities of Diverse Culture Broths of *Lactobacillus Sakei* Probio65

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Abstract

Lactobacillus sakei probio65 is a bacteriocinogenic lactic acid bacteria, capable of inhibiting the growth of microbial pathogens mainly from enterobacteriaceae family. In order to optimize bacteriocin production without the use of Soya peptone in media, as it is found to be an allergen for many people, this study evaluated the effect of MRS-SP enriched culture broths towards *Corynebacterium tuberculostearicum*, *Staphylococcus aureus* and *Listeria monocytogenes*. Optical density measurements were the principle parameter to assess the growth of the p65 in varios formulations. Different yeast extract, amino acid and vitamin mixture concentrations were taken into consideration while optimizing the media. MRS-SP supplemented with 0.4% of amino acid mixture, 0.4% cysteine, 0. 002% vitamin C showed promising results for inhibiting the given pathogens both in CFS and CCB state with average zone of inhibition from 10-13mm by disc diffusion method. pH regulated to 7.0 at inoculation during the use of the amino acids and after 20:00h of incubation at 37 $^{\circ}$ C, found to be around 4.00 with high anti-pathogenic activity. *Lactobacillu. sake*i P65 is widely used in South Korea, Japan and China in biopharmaceutical and dairy industry, in order to specifically detect it within mixed strains. By whole genome sequencing four novel genes were identified and a specific primer is designed for one of them

Keywords: Pathogens, anti-pathogenic activities, Probio65, whole genome sequencing

Introduction

With alarming rise of antibiotic resistance, there are less adequate alternatives which can replace the antibiotics; probiotics can be used as an alternative for it. The undergoing research upon the mechanism of the antibiotic resistance proves that the consumption of wholly antibiotics can mimic the cell transcriptional as well as translational mechanisms, thus proves to be fatal for the host cell, but the use of probiotic strains can overcome this problem. Further the richness in gut microflora can also overcome it and the richness can

be enhanced by the use of the probiotics. Nowadays, probiotics have been used in pharmaceuticals, cosmetics as well in dairy industries, so that a common man's gut microflora gets boosted. Probiotics usually found in fermented foods have been optimised at large scale fermentations so to get the viable count. The probiotic concept is open to lots of different applications in a large variety of fields relevant for human and animal health. The oral consumption of probiotic microorganisms produces a protective effect on the gut flora (Tannock, 1988). Many clinical studies have proven the effectiveness of probiotics for treatment of diseases such as treatment of traveller's diarrhoea, antibiotic associated diarrhoea and acute diarrhoea obesity, insulin resistance syndrome, type 2 diabetes, and non-alcoholic fatty liver disease, Furthermore, the positive effects of probiotics on human health have been demonstrated by increasing the body's immunity (immunomodulation) (Gismondo *et al.,* 1999). To date, studies suggest that probiotics may have a clinical prophylactic application in various therapies such as anticancer (Chen and Khismatullin, 2014), antioxidant, antiallergy (Lee *et al.,* 2014), cholesterol-lowering (Guo *et al.,* 2015) and antidiabetic treatments (Chen *et al.,* 2014; Giraffa, 2012).The prophylactic effects observed were dependent on the type of probiotic strain studied. It has been suggested that probiotics may exert an anticancer effect by decreasing the influence of chemical carcinogens by the following means: (1) the detoxification of ingested carcinogens; (2) the alteration of the environment of the intestine, which decreases the populations or metabolic activities of bacteria that may generate carcinogenic compounds; (3) the induction of apoptosis via the production of metabolic products such as butyrate; (4) the production of compounds that inhibit the growth of tumor cells; and (5) the stimulation of the immune system (Lee *et al.,* 2011; Parvez *et al.,* 2006; Uccello *et al.,* 2012). Molecular and genetic studies allowed the determination of the basics of the beneficial effect of probiotics, involving Antagonism through the production of antimicrobial substances (Vandenbergh, 1993), Competition with pathogens for adhesion to the epithelium and for nutrients (Gulliot, 2003), immunomodulation of the host (Isolauri *et al.,* 2001), Inhibition of bacterial toxin production (Brandao *et al.,* 1998).

The ability of probiotic strains to co-aggregate, as one of their mechanisms of action, may lead to the formation of a protective barrier preventing pathogenic bacteria from the colonisation of the epithelium, thus blocking pathogens (Schachtsiek *et al.,* 2004) and may trigger a signalling cascade, leading to immunological modulation (Oelschlaeger, 2010). The last of the above mentioned probiotic effects-inhibition of the production of bacterial toxins-is based on actions leading to toxin inactivation and help with the removal of toxins from the body (Schatzmayr *et al.,* 2006; Nikabakht Nasrabadi *et al.,* 2013). The use of a combination of probiotics demonstrating various mechanisms of action may provide enhanced protection offered by a bio-therapeutic product (Lima-Filho *et al.,* 2000).

Material and methods

Bacterial cultures and growth curve

Lacobacillus sakei probio65 and other bacterial pathogens were taken from the microbiome laboratory of Yeungnam University South Korea. P65 was grown in commercial MRS media (MB Cell, Korea). Bacterial pure cultures were maintained in MRS broth (B Cell, Korea) and stored at -80°C in MRS broth with 20% (v/v) sterile glycerol. All strains used in this study were incubated at 37°C and sub cultured at least twice before use. MRS (DeMan Rogosa and Sharpe) broth 0.5X was used for studying the growth curve of *L.sakei* probio65, taking 100 µl of p65 from stock and inoculating it into 10 ml of MRS broth tube, incubated at 36 °C for 20:00 h to form freshly prepared seed culture. After 20:00 h, 100 µl from seed culture was separately inoculated into 07 MRS tubes (10 ml each), incubated at 36 °C degrees , after every 4:00 h, optical density (600 nm) was measured with dilution of 20X. .

Media formulation for p65 – MRS and MRS-SP

Soya peptone is found to be a great enhancer of growth for the p65, as it is having a lot of amino acids in it, as soya peptone shown some allergies in some subjects, taking into that consideration, the experiments were done to replace it with the single amino acid or a group of amino acids. Varied concentrations of yeast extract; 1%, 0.5%, 0.25%, 0.10%, 0.05% with base composition of the MRS-SP also used in evaluating the effect of it on growth as well. With 0.1% yeast extract along with 0.4% of the amino acid mixture taken to formulate the new media M2, M1 taken as the control group. Same with both 0.4% and 0.8% amino acid mixture along with the addition of crude vitamin mixture (100µl) and vitamin C (0.002%) as well. Effect of vitamin mixture was evaluated by using it in three media formulations with 100µl and 10µl, in order to check the sole impact of vitamin mixture, thus media without yeast extract was also formulated with 100µl of the vitamin solution (**Table 1**).

MRS-SP enriched with classified and single amino acids.

With base composition of MRS-SP and 0.1% yeast extract enriched with classified amino acids hydrophobic, hydrophilic, acidic and basic, two concentrations of those were selected 0.2% and 0.4% (**Table 3**), thus making eight media formulations with it. Singly hydrophilic and acidic amino acids with concentrations of 0.2% and 0.4% taken making 14 media formulations as well (**Table 2**).

Inoculation and optical density measurements

All the media's were inoculated with freshly prepared p65 (20:00h) grown in MRS (commercial). With dilution of 20X, the OD was measured with UV spectrophotometer (Hitachi U-2000) at 600nm.

pH measurements

Before inoculation, the pH was regulated to 6.5-7.00 with NaOH or with glacial acetic acid, before and after the pH was recorded.

Evaluation of anti-pathogenic potential of *Lactobacillus sakei* probio65 against various pathogens

Cell culture broth (CCB) and cell free supernatant (CFS); cell culture broth is wholly grown culture with media while as cell free supernatant is the centrifuged supernatant of the cell culture, of various formulated medias have been used via paper disc diffusion method in order to check the anti-pathogenic activity of diverse culture broths of p65 against *Corynebacterium tuberculostearicum*, *Staphylococcus aureus* and *Listeria monocytogenes*.

Corynebacterium tuberculostearicum:- 10µl of the freshly grown pathogen was cultured on R-agar (Bactopeptone-10g, yeast extract-5g, malt extract-5g, casamino acid-5g, beef extract-2g, glycerol-2g, tween 80-5g, $MgSO_4.7H_2O$-1g, agar-15g, H_2O-1litre) and the sterilized paper discs upon it loaded with 100µl of CCB and CFS, then incubated at 37°C in CO_2 incubator for 12:00 h

Staphylococcus aureus:- 10µl of the freshly grown pathogen was cultured on tryptic soy agar (B Cell, Korea) and the sterilized paper discs upon it was loaded with 100µl of CCB and CFS of various formulations, then incubated at 37°C for 12:00h

Listeria monocytogenes:-. 10µl of the freshly grown pathogen was cultured on nutrient agar (B-Cell, Korea) and the sterilized paper discs upon it loaded with 100µl of CCB and CFS of various formulations, then incubated at 37°C for 12:00 h.

Table 1: Media formulations for p65

Media name	Yeast extract	Soya peptone	Sodium acetate	Dipotassium phosphate	Ammonium sulphate	Magnesium sulphate	Manganese sulphate	Tween 80	Glucose	Amino acid mixture	Vitamin mixture(µl)	Vitamin C %
SM1	1%	-	0.5%	0.2%	0.2%	0.01%	0.005%	0.1%	2%	-	-	-
SM2	+	0.5%	+	+	+	+	+	+	+	-	-	-
MYE-1	1%	-	+	+	+	+	+	+	+	-	-	-
MYE-0.5	0.5%	-	+	+	+	+	+	+	+	-	-	-
MYE-0.25	0.25%	-	+	+	+	+	+	+	+	-	-	-
MYE-0.1	0.1%	-	+	+	+	+	+	+	+	-	-	-
MYE-0.05	0.05%	-	+	+	+	+	+	+	+	-	-	-
M1	0.1%	-	+	+	+	+	+	+	+	-	-	-
M2	+	-	+	+	+	+	+	+	+	0.4%	-	-
B1	+	-	+	+	+	+	+	+	+	+	-	-
B2	+	-	+	+	+	+	+	+	+	+	100	-
B3	+	-	+	+	+	+	+	+	+	+	-	0.002
B4	+	-	+	+	+	+	+	+	+	0.8%	100	-
N1	+	-	+	+	+	+	+	+	+	0.4%	-	-
N2	+	-	+	+	+	+	+	+	+	+	100	-
N3	+	-	+	+	+	+	+	+	+	+	10	-

N4	-	-	+	+	+	+	+	+	+	+	100	-

Table 2: MRS-SP with classified and single amino acids concentrations

Media name	Base media	Amino acid used	Amino acid concentration (%)
U1	MYE-1	Hydrophobic	0.2
U2	+	+	0.4
U3	+	Hydrophilic	0.2
U4	+	+	0.4
U5	+	Acidic	0.2
U6	+	+	0.4
U7	+	Basic	0.2
U8	+	+	0.4
P1	+	Tyrosine	0.4
P2	+	Tyrosine	0.2
P3	+	Aspartic acid	0.4
P4	+	Aspartic acid	0.2
P5	+	Serine	0.4
P6	+	Serine	0.2
P7	+	Glutamic acid	0.4
P8	+	Glutamic acid	0.2
P9	+	Cysteine	0.4
P10	+	Cysteine	0.2
P11	+	Threonine	0.4
P12	+	Threonine	0.2
P13	+	Glutamine	0.4

P14	+	Glutamine	0.2

Table 3: Classified amino acid concentrations

Hydrophobic amino acids		
Amino acid	Group1-Conc. (0.005%)	Group2-Conc. (0.10%)
Glycine	+	+
Proline	+	+
Methionine	+	+
Tryptophan	+	+
Total	0.2%	0.4%
Hydrophilic Amino acids		
Amino acid	Group1-conc. (0.005%)	Group2-conc. (0.001%)
Serine	+	+
Threonine	+	+
Glutamine	+	+
Tyrosine	+	+
Total	0.2%	0.4%
Acidic Amino acids		
Amino acid	Group1-conc. (0.067%)	Group2-conc. (0.14%)
Glutamic acid	0.067	0.14
Aspartic acid	0.067	0.14
Cysteine	0.067	0.14
Total	0.2%	0.4%
Basic Amino acids		
Amino acid	Group1-conc. (0.067%)	Group2-conc. (0.14%)
Lysine	+	+
Arginine	+	+
Histidine	+	+
Total	0.2%	0.4%

Results

Growth curve of p65

Growth curve of the *L.sakei* probio65 (**Figure 1**) shows there is progressive increase in the OD but after it, it gets decreased, which is an indication that stationary phase has started. There is little or almost negligible lag phase in this graph, as inoculum was taken from the seed culture in a state of exponential phase. All the cultures used were incubated for 20:00h in 37°C.

Impact of soya peptone on the growth of p65.

Growth of p65 in MRS (SM-2) and MRS-SP (SM-1) was measured, so to confirm the impact of soya peptone on growth, found to be more in media (SM-2) that contain soya-peptone than media (SM-1) that lacks it (**Table 4).**

Growth of p65 in MRS-SP with varied concentrations of yeast extract.

Yeast extract concentration having a proportional impact on the growth of the p65. With decreasing concentrations of yeast extract, there is a decrease in the growth i.e optical density of the p65 (MYE-1, MYE-0.5, MYE-0.25, MYE-0.10, MYE-0.05) from 5.56 to 0.56 (**Table 4**).

Growth of p65 in MRS-SP and MRS-SP enriched with amino acid mixture, crude vitamin mixture and vitamin C.

With addition of amino acid mixture, amino acids; L- Glutamic acid, L-Lysine, L-Methionine, L-Proline, L-Serine, L-Glutamine, L-Cysteine, L-Threonine, L-Tryptophan, L-Histidine, L-Arginine, L-Tyrosine, L-Aspartic acid, L-Asparagine, L-Glycine, all with 0.60g, taking 0.4% of it in base media MRS-SP (M1), after 20:00h OD_{600} measured is 1.84, while in MRS-SP without amino acid mixture, OD_{600} measured is 0.80. Effect of two concentrations of amino acids i-e 0.4% and 0.8% along with the addition of the Vitamin mixture and vitamin C as well. There is an increase in growth, not when the concentration of amino acid mixture is 0.8%, so there are some amino acids in the mixture whose concentration may retard the growth very much. In order to validate the effect of the vitamin mixture, media N2, N3, N4 with 100µl, 10µl and 100µl, not the N4 with yeast extract. OD_{600} varied from 2.16 to 0.24, with N2 as the highest (**Table 4**). In broth N2 pH falls from 7.0 to 4.0, after which N3 and N1 with 4.4 and 4.6 respectively, thus vitamins play a great role in the pH regulation as well (**Table 5**).

Growth of p65 in classified and single amino acid enriched MRS-SP media.

With advent of classified amino acids in two concentrations, OD_{600} varied from 0.82 to 1.16, with U4 as highest, that is MRS-SP with hydrophilic amino acid mixture of 0.4%, afterwards 1.08 in same base media enriched with 0.4% of acidic amino acid mixture, U6. In both broths, the pH was dipped to 04 (**Table 5**), as long as there is a decrease in pH of the media, there will be an increase in optical density but it should be analysed that is this increase only in the biomass or should it have some impact on the viable cell count as well. In single amino acid enriched culture broths, OD_{600} lies in between 0.83 to 1.50. Highest in P7 with 0.4% glutamic acid, followed by 0.2% of glutamic acid enriched media with 1.25, 0.2% tyrosine and 0.4% serine enriched media also showed promising growth as 1.22 and 1.15 respectively **(Table 7**). pH after 20:00h of incubation reached to 4.00 (**Table 5**).

Verification of zone of inhibition by optimised culture broths of *L.sakei* P65.

Fourteen optimised culture broths were used for checking the anti-pathogenic activity against the given pathogens. For *Corynebacterium tuberculostearicum*, the best result was shown by CFS of broth with base composition of MRS-SP, yeast extract conc. 0.1% and amino acid concentration 0.4% that is media M2/N1. The zone of inhibition by M2/N1 for *Corynebacterium tuberculostearicum* is 13mm (**Figure 2**), which is more than that of the commercial MRS cultured broth. Broths enriched with acidic mixture of 0.4% (M2/N1), cysteine 0.4% (P9) and 0.002% vitamin C (B3) separately show the zone of inhibitions for *Staphylococcus aureus* both in CCB and in CFS with 10mm in trios (**Figure 3**). Culture broths enriched with 0.4% acidic amino acid (M2/N1), 0.002% vitamin C (B3) shows zone of inhibition for *Listeria monocytogenes* in CCB state with 12mm (**Figure 4)**, more than that of MRS cultured broth.

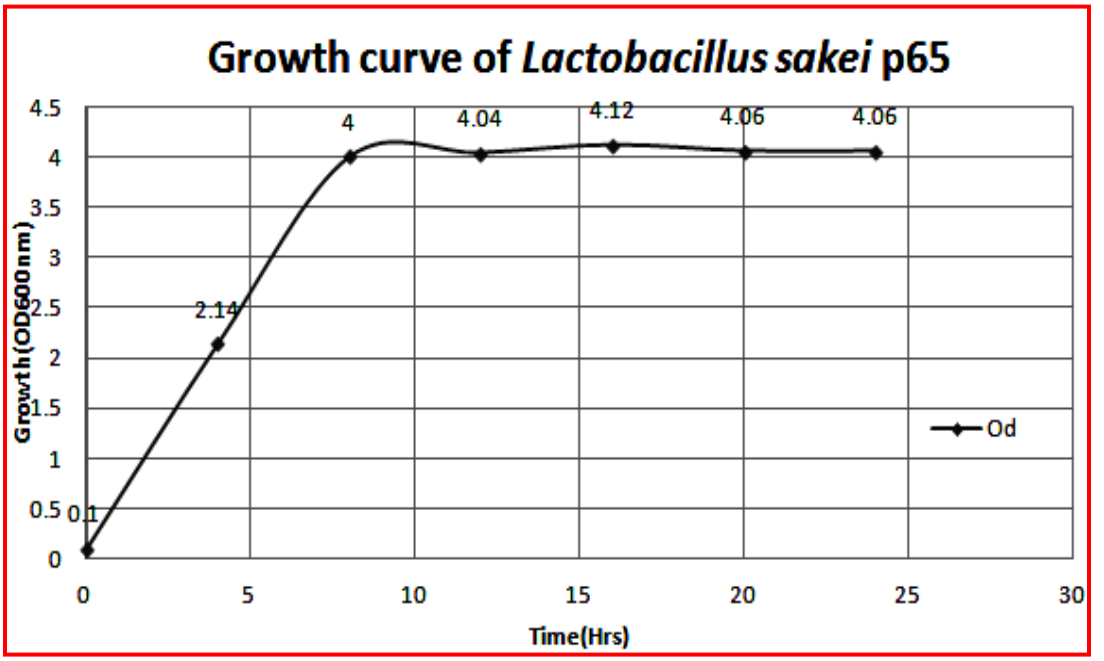

Figure 1: Growth curve of p65

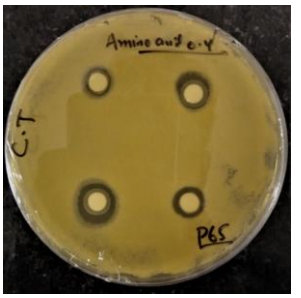

Figure 2: Zone of inhibition by optimised p65 culture broth against *Corynebacterium tuberculostearicum*

Figure 3: Zone of inhibition by optimised culture broth of p65 on *Staphylococcus aureus*

Figure 4: Zone of inhibition by optimised culture broths of p65 against *Listeria monocytogenes*

Table 4: Optical density measure of p65 in various media formulations

Broth	OD_{600} 0:00h (X20)	OD_{600} 20:00h (X20)
SM1	0.46	6.16
SM2	0.40	6.50
MYE-1	0.46	5.56
MYE-0.5	0.40	3.50
MYE-0.25	0.36	1.80
MYE-0.10	0.30	0.90
MYE-0.05	0.22	0.56
M1	0.28	0.80
M2	0.22	1.84
B1	0.20	1.82

B2	0.26	2.20
B3	0.22	1.64
B4	0.20	1.84
N1	0.24	1.38
N2	0.22	2.16
N3	0.22	1.6
N4	0.18	0.24

Table 5: pH measurements of broths at varied time intervals

Broth	pH 0:00h	pH 20:00h
SM1	6.8	4.2
SM2	7.0	4.5
MYE-1	7.2	4.6
MYE-0.5	7.4	4.3
MYE-0.25	7.4	4.7
MYE-0.10	7.4	4.6
MYE-0.05	7.2	4.8
M1	7.0	3.9
M2	7.4	4.6
B1	7.4	4.6
B2	7.4	4.2
B3	7.3	4.0
B4	7.2	4.4
N1	7.4	4.6
N2	7.0	4.0
N3	7.0	4.4
N4	7.6	5.6
U1	7.0	4.0
U2	7.5	4.3
U3	7.2	4.0

Broth	pH 0:00h	pH 20:00h
U4	7.4	4.0
U5	7.2	4.0
U6	6.7	4.0
U7	7.8	4.3
U8	8.2	5.4
P1	6.8	4.0
P2	7.4	4.0
P3	6.7	4.0
P4	7.2	4.0
P5	7.0	4.0
P6	7.0	4.0
P7	6.6	4.0
P8	7.0	4.0
P9	7.0	4.0
P10	6.6	4.0
P11	7.0	4.0
P12	7.0	4.0
P13	7.2	4.0
P14	7.0	4.0

Table 6: Measure of zone of inhibition by p65 against various pathogens.

Culture broth	Pathogen	CFS	CCB	Measure of Zone (mm)
Commercial MRS	*Corynebacterium tuberculostearicum*	+	-	10
M2/N1	*Corynebacterium tuberculostearicum*	+	-	13
Commercial MRS	*Staphylococcus aureus*	+	+	13
M2/N1	*Staphylococcus aureus*	-	+	10
P9	*Staphylococcus aureus*	-	+	10
B3	*Staphylococcus aureus*	-	+	10
Commercial MRS	*Listeria monocytogenes*	-	+	10
B3	*Listeria monocytogenes*	-	+	12

Table 7: Optical density measure of p65 in classified and single amino acid enriched media

Broth	OD_{600} 0:00h (X20)	OD_{600} 20:00h (X20)
U1	0.36	1.00
U2	0.38	0.82
U3	0.18	0.94
U4	0.36	1.16
U5	0.28	0.86
U6	0.30	1.08
U7	0.18	0.82
U8	0.30	0.94
P1	0.32	1.11
P2	0.31	1.22
P3	0.28	1.00
P4	0.18	0.98
P5	0.18	1.15
P6	0.20	1.10
P7	0.38	1.50
P8	0.18	1.25
P9	0.18	1.00
P10	0.20	1.00
P11	0.36	0.98
P12	0.38	0.83
P13	0.32	0.96
P14	0.38	0.98

Discussion

The *Lactobacillus sakei* probio65 cultivated in various culture broths have been tested for its antibacterial activity against the *Corynebacterium tuberculostearicun*, *Staphylococcus aureus* and *Listeria monocytogenes* which showed strong antibacterial activity. This activity is due to the production of the organic acids like acetic acid and lactic acid. Organic acids have strong inhibitory action against the pathogens, as the un dissociated organic acid enters the bacterial cell and dissociates inside the cell cytoplasm, this eventually drips down the pH and there will be accumulation of the ionized forms of organic acids which destroys the pathogen (Leftris Makras and Luc Du Vyust, 2006). Nevertheless it is found that *L.sakei* p65 produces the antibacterial substances different from organic acids. The CFS of the *L.sakei* p65 is found to be

strong against the pathogens and it is also reported in the Bifidobacterium strains as well which are used as probiotics (Russel and Diez, 1998). Lactic acid bacteria are nutritionally fastidious micro-organisms, with a high requirement for pre-formed amino acids (Morishita *et al.,* 1981; Chopin 1993). In present study, the amino acids were used in two concentrations that is 0.4% and 0.2%, so to check the impact of it on the growth of the p65. The extracellular protease products of the lactic acid bacteria are localized and usually peptide products of (4±8 amino acids) are imbibed by the cell. (Kunji *et al.,* 1996). Aminopeptidases hydrolyse peptides and the amino acids which are not required for growth are released into the medium (Van Boven and Konigs, 1988). Taking into that consideration, various amino acid combinations or single amino acids were screened with base media MRS-SP towards the growth. Strains of *Lact. curvatus*, *Lact. brevis*, *Lact. buchneri*, *Lact. plantarum* and *Lact. casei* catabolize arginine (Liu *et al.,* 1995; Straub *et al.,* 1995). In present study, 0.4% of acidic and hydrophilic amino acid mixture enriched media showed good measure of the OD_{600} with pH 4.00 after 20:00h of incubation at 37 ^{o}C as same of *L. plantarum* JNU 2116, isolated from kimchi, has a high survival rate at low pH (Heeseop Yoo *et al.,* 21018). With low levels of the nutrient levels, there will be the high levels of the bacteriocin production as in *lactis* strain of *L. lactis* subsp. with low nutrient concentration supported a higher relative specific nisin production rate compared with one cultivated with higher nutrient concentrations (Kim *et al.,* 1997) due to the stress conditions, same taken into consideration as well. The anti-pathogenic activity of the culture broths with minimal nutritional constituents showed the zone of inhibitions more than that of the commercial MRS cultured broth with 13mm for *Corynebacterium tuberculostearicum* and 12 mm for the *Listeria monocytogens*. In rich environment cells reduce their defensive bacteriocin production mechanism, underlying the potential role of bacteriocin production in natural, competitive, and nutrient-depleted ecosystems. An increase of the yeast extract content of a tryptone medium increased the specific bacteriocin production by *L. sakei* CCUG 42687 (Aasen *et al.,* 2000), whereas alteration of the nitrogen source of MRS broth did not seem to affect the plantaricin production rate by *Lactobacillus plantarum* TMW1.25 (Klostermaier *et al.,* 1990). In view of practical applications, it seems to be of crucial importance that the bacteriocin-producing strain is perfectly adapted to the nutrient environment. *L. sakei*

CTC 494, isolated from naturally fermented sausage (Hugas *et al.,* 1995) produces high amounts of bacteriocin in a meat-like environment (MRS broth), but bacteriocin production completely fails in a casein tryptone medium (Leroy and Vuyst, 1999). The latter medium does not offer a nutrient spectrum that permits good cell growth, nor does it support bacteriocin production. In conclusion, extremely rich environments will not necessarily lead to a gain of bacteriocin activity but if one get some hypersensitivity, then screening of that very constituent like soya-peptone in that study can be replaced with the other one, it should be specific.

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