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Full Length Article

Isolation, *In Vitro* Probiotic Characterization of *Lactobacillus plantarum* and its Role on Italian ryegrass Silage Quality Enhancement

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Abstract

Italian ryegrass (IRG) is an important forage type for feeding ruminants. IRG has difficult to ensile with good quality. Hence the inoculation IRG with lactic acid producing bacterial strains (LAB) gives an alternate solution to produce quality silage. Accordingly new Lactobacillus sp. KCC-32 was isolated from fermented animal manure. Biochemical and physiological studies showed that the strain belonged to Gram positive, produced gas from glucose and catalase-negative. The 16S rRNA sequence analysis revealed that KCC-32 showed 99% similarity towards *Lactobacillus plantarum* sp. Further, KCC-32 displayed potential probiotic characteristics including resistant to low pH, bile salt tolerance, auto-aggregation and hydrophobicity. The homo fermentative activity of KCC-32 resulted in the enhancement of Italian ryegrass silage quality. In addition, KCC-32 added silage group showed significantly ($P \leq 0.05$) increased lactic acid production (4.891 DM%) and the nutrient profile resulted with high crude protein content with less acid detergent fiber (ADF) and neutral detergent fiber (NDF) percentage when compared to control group. Further, the microbial count of KCC-32 silage group displayed significantly ($P \leq 0.05$) high LAB count (24.3×10^7 cfu/g) and no fungal as well as yeast growth. Hence, this study suggests that KCC-32 has potential probiotic characteristics and the addition of KCC-32 to the IRG silage can improve the fermentation quality for the production of high-quality silage. © 2017 Friends Science Publishers

Keywords: Antifungal; Lactic acid; Silage; ADF; NDF; Probiotic

Introduction

Probiotics are the viable microorganism that provides beneficial effects on the host health by maintaining and improving the intestinal microbial growth (Linda *et al*., 2015). Such probiotics possess various physiological functions such as assisting digestion, inhibition of pathogens, and antitumor activity (Clarke *et al*., 2012). The potential probiotic strain contains resistance to gastric stress and bile salt environments present in the gastrointestinal tract, more adhesion properties into the epithelial cells, and control pathogen adhesion using specific competition (Argyri *et al*., 2013; Hill *et al*., 2014). Further, probiotic can act as an antimicrobial agent, and various probiotic strains are reported to enhance the immune response activity of natural killer cells (Mera *et al*., 2012).

Probiotic strains are identified as safe because of their extended use in fermented food. Probiotic bacteria help food fermentation by various ways including (i) preservation by lactic acid production which act as antimicrobial agent (Fraqueza, 2015); (ii) The production of flavor compound that will provide the organoleptic properties (Smid and Kleerebezem, 2014); (iii) Improvement of nutritional value of the food; (iv) production of therapeutic agent and control of serum cholesterol (Nuraida, 2015). Adhesion of the probiotic strain to intestinal mucosa is considered as the main selection principle for probiotics (Ramos *et al*., 2013) as it sustains in the intestine for the long period and thus provides beneficial effects longer to the host (Mehmet *et al*., 2015). Among various probiotic strains, *Lactobacillus plantarum* has a significant role in food fermentation and health promoting properties.

Ensilation is the process that used for preserving forage crops. Silages are preserved forage crops that are preserved using ensilation process in many countries including Korea, China and Japan. During ensilation the inhibition of undesirable microbial growth mainly depends on sufficient organic acid production (Cai *et al*., 1999).

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To achieve high organic acid production, homo and hetero fermentative lactic acid bacteria (LAB) are used as additives. In this process LAB change the carbohydrates into organic acids. Lactic acid is the main organic acid that is produced during silage fermentation (McDonald *et al*., 1991).

Lactic acid is responsible for the acidic nature of silage and inhibits the growth of undesirable microorganisms and plant enzymatic activities. Among homo and heterolytic fermentation, homolytic fermentation is considered more desirable because of high recovery of dry matter and energy. *Lactobacillus* sp. plays significant role in homolytic fermentation. Further, the *Lactobacillus* sp. mainly used in the fermentation of dairy products and preservation (Giraffa *et al*., 2010). *Lactobacillus* strains have been used as starters in the protection of fermented vegetables, dairy foods and fish for decades. Thus adding potential *Lactobacillus* inoculants to silage increasing the fermentation and thereby producing high quality silage (Avila *et al*., 2010). On the other hand, adding *Lactobacillus* to silage has numerous benefits including non-corrosive to farm machinery, less cost than enzyme formulation and do not pollute the environment (Weinberg *et al*., 2003). Hence, the objective of this study has related to determine the *in vitro* probiotic characteristic of KCC-32 and its role in the enhancement of IRG silage quality.

Materials and Methods

Sample Collection, Biochemical Characterization and Anti-fungal Analysis

The animal manure samples were collected from Cheonan, South Korea. One gram of the sample was serially diluted using sterile water. Based on antifungal results, an effective strain was selected for further characterization and the strain was named as KCC-32. An overnight culture of KCC-32 was used for the analysis of biochemical and physiological properties. Megazyme assay kit (Bray, Co. WIcklow, Ireland) was used in the quantification of fermentative acid. The API 50 CH and API-ZYM kits (Marcy-I' Etoile, France) were used for the analysis of carbohydrate fermentation and enzyme production. The screening of antibiotic sensitivity was tested by disc diffusion method (Valan Arasu *et al*., 2013).

16s rRNA Sequencing and Gene Bank Deposition

The 16s rRNA gene sequencing was carried out at the Solgent Co (Seoul, South Korea) by the method of (Sanger *et al*., 1977). The genomic DNA of KCC-32 was isolated and purified by QIAquick® kit (Qiagen Ltd., Crawley, UK). The amplicons were sequenced using universal primers 27 F (5' AGA GTT TGA TCG TGG CTC AG 3') and the 1492 R (3' GCT TAC CTT GTT ACG ACT T 5'). The aligned 16srRNA sequence of the KCC-32 was subjected to BLAST with the non-redundant database of the NCBI GENE BANK. Further, the obtained 16s rRNA sequence of the isolate KCC-32 was deposited into NCBI Genebank.

Experiment on Low pH and Bile Salt Tolerance of KCC-32

The low pH tolerance and gastric juice resistance of KCC-32 were analyzed using the protocol described by (Charteris *et al*., 1998). For bile resistant analysis, fresh culture of KCC-32 was inoculated into the sterilized MRS broth containing bile salts such as 0.5% sodium deoxycholate and 0.3% oxgall (DCA Sigma, St Louis, MO, USA) and incubated at 37℃. 200 µL of the samples were taken after 24 h and 48 h respectively. Optical intensity was read at 600 nm.Without bile salts treated as control.

Auto Aggregation and Hydrophobicity Analysis of KCC-32

The aggregation (Del Re *et al*., 2000) and hydrophobicity (Rosenberg *et al*., 1980) were measured.

Sample Collection and Silage Making

Early stage of Italian ryegrass IRG was collected at, NIAS-RDA, Jeju. Two sets of 200 g of IRG were weighed. One set was served as control and the other was inoculated with (1.5×10^{10} cfu/g) KCC-32. Subsequently, the air inside the silage bag was removed and sealed. Similarly, triplicate samples were prepared and stored in room temperature for 45 days. After 45 days, the bags were opened, silage nutrient profile, and microbial content were analyzed.

Silage Nutritive Profile Analysis

The silage nutritive profile such as crude protein (CP: AOAC, 1990), Neutral detergent fibre (NDF: Van Soest *et al*., 1993), Acid detergent fibre (ADF: Van Soest *et al*., 1993), and Total digestible nutrient (TDN: Holland *et al*., 1990; Seo *et al*., 2010) were estimated using standard protocols.

Microbial Profile Analyzes

Serial dilution was made according to the method described by (Miller and Wolin, 1974). 100 µL of sample was spread on MRS specific media for Lactic acid bacteria and incubated at 28±1℃ for 48 h. 3 M petriflim (3 M Microbiology products, St. Paul, USA) was used for the counting of yeasts and molds. Potato dextrose agar (Difco) was used for the fungal population counting.

Estimation of Organic Acids

About 10 g of silage sample after 45 days of incubation was mixed with 90 mL distilled water and stored at 4℃ for 24 h. Firstly, samples were filtered through the filter paper

(Whatman No. 6) and the filtrate was re-filtered using 0.22 µm syringe filter before injection of samples in HPLC (HP1100., Agilent USA). The combination electrode was used to calculate the pH of the sample. The filtrate was stabilized with 5% meta-phosphoric acid and the filtrate was stored at -70℃. The organic acids such as lactic acid (HPLC), and acetic acid, butyric acid (Gas chromatography; GC-450, Varian Co., USA) were analyzed (Kristensen *et al*., 2007).

Evaluation of Statistics

All samples were evaluated in three replicates. SPSS/PC (v. 12.0) package was used for the analysis of variance of all the variables. The Duncan's multiple range test were used to determine the treatment mean difference at 5% probability level.

Results

Isolation and Characterization of KCC-32

In this study, 15 LAB strains were isolated from animal manure. Among these isolated strains, single strain showed potential result in preliminary tests. Then the strain was named as KCC-32. Hence, the strain has been subjected to further experimental analysis. The 16 srRNA sequencing of the selected strain was blast with NCBI nucleotide sequence database. The results demonstrated more similarity with *Lactobacillus plantarum* (≥99% similarity). Hence, we confirmed that KCC-32 belonged to *Lactobacillus plantarum*. The 16srRNA sequence of KCC-32 was assigned accession number KP091748.1 at NCBI Genebank.

Physiological and Biochemical Characterization of KCC-32

The isolated *Lactobacillus plantarum* KCC-32 was subjected to various biochemical and probiotic experiments. KCC-32 was grown in MRS broth for 48 h. After 48 h of growth, the microscopical observation resulted that KCC-32 was creamy in color, rod-shaped and Gram positive. The carbohydrate utilizing property of KCC-32 showed the fermentation of various carbohydrates (Table 1). The enzyme production analysis of KCC-32 confirmed the production of different intra and extra cellular enzymes (Table 2). Also, KCC-32 showed susceptibility to the common antibiotics (Table 3). Estimation of fermentative product of KCC-32 resulted majorly with lactic acid (127.23 µg/mL), acetic acid and Succinic acid respectively (Table 4).

Resistant Analysis of *L. plantarum* KCC-32 against Low pH and Bile Salts

The ability of KCC-32 to grow in GI tract conditions like low pH, resistant to bile salts was analyzed. The results confirmed that KKC-32 can survive in the GI tract conditions like low pH (Fig. 1A), tolerance towards induced gastric juice stress (pH 2 and pH 3) condition (Fig. 1B). No live cells were noticed below pH 2. Subsequently, KCC-32 displayed potential resistance against the toxic bile salts such as oxgall (0.3%) and sodium deoxycholate (0.5%) (Fig. 1C).

Auto Aggregation and Cell Surface Hydrophobicity Analysis of *L. plantarum* KCC-32

The auto-aggregation and hydrophobicity experiments were carried out to find the ability of KCC-32 to form colonies inside the gut. In this experiment, KCC-32 showed strong auto-aggregation activity and the rate of auto-aggregation increased with the increase in time (Fig. 2B). Further, the cell surface hydrophobicity experiment was carried out using chloroform and xylene. The results revealed that KCC-32 showed strong hydrophobicity towards xylene with 59.07% (Fig. 2A).

IRG Silage Quality and Nutrient Composition Analysis

The IRG silage nutrient parameters including ADF, CP, TDN and NDF in control and experimental group were studied. A notable improvement in crude protein level of KCC-32 treated group was noted when compared to control IRG silage group. Simultaneously there was moderate decrease in ADF and NDF was noted as compared to control group. But in case of total digestible nutrient (TDN), slight increase was noted in KCC-32 inoculated group as compared to normal control group (Table 5). The microbial population profile of control and experimental group was listed in (Table 6). Significant ($P \leq 5$) increase in LAB population was noted in KCC-32 inoculated group as compared to control group. Further, no yeast and fungal growth was noted in the KCC-32 treated experimental group when compared to normal control group. The level of organic acids produced during IRG silage ensilation process is listed in (Table 7). There was significant ($p<0.05$) increase in lactic acid production was noted in KCC-32 inoculated group as compared to control group. Further, slight increase in acetic acid level and no butyric acid production was noted in KCC-32 treated group as compared to control group.

Discussion

Among various probiotic strains, *L. plantarum* attracted more researchers worldwide because of its unique application in clinical as well as industrial needs. The character of *L. plantarum* to adopt different environments and various beneficial applications makes *L. plantarum* especially interesting and challenging. The most striking feature of *L. plantarum* was the high potential to import and metabolise a large number of carbohydrates (Alena *et al*., 2015). Accordingly, in this study the *L.*

Table 1: Biochemical characterization of isolated *L. Plantarum* KCC-32using API 50 CHB system

Name of the carbohydrates	KCC-32
Glycerol	-
Erythritol	+
D-Arabinose	+
L-Arabinose	+
D-Ribose	+
D-Xylose	+
L-Xylose	+
D-Adonitol	-
Methyl-βD-Xylopyranoside	-
D-Galactose	-
D-Glucose	+
D-Fructose	+
D-Mannose	-
L-Sorbose	+
L-Rhamnose	+
Dulcitol	+
Inositol	+
D-Mannitol	+
D-Sorbitol	+
Methyl-αD-Mannopyranoside	+
Methyl-αD-Glucopyranoside	+
N-Acetylglucosamine	-
Amygdalin	+
Arbutin	-
Esculinferric citrate	+
Salicin	+
D-Cellobiose	+
D-Maltose	-
D-Lactose	+
D-Melibiose	+
D-Saccharose	-
D-Trehalose	+
Inulin	+
D-Melezitose	+
D-Raffinose	+
Amidon	-
Glycogen	+
Xylitol	+
Gentiobiose	+
D-Turanose	+
D-Lyxose	+
D-Tagatose	+
D-Fucose	-
L-Fucose	-
D-Arabitol	-
L-Arabitol	-
Potassium gluconate	+
Potassium2-keto gluconate	-
potassium 5-keto gluconate	+

(+): Positive response; (-): Negative response

Table 2: Analysis of intra and extra cellular enzyme production from KCC-32

Extracellular enzymes	KCC-32
Alkaline phosphatase	++
Esterase (C_4)	++
Esterase lipase (C_8)	+++
Lipase (C_{14})	++
Leucine arylamidase	+++
Valine arylamidase	++
Cystine arylamidase	++
Trypsin	++
α-Chymotrypsin	+++
Acid phosphatase	+
Naphthol-AS-biphosphohydrolase	++
α-Galactosidase	+
β-Galactosidase	++
β-Glucuronidase	+++
α-Glucosidase	++
β-Glucosidase	++
N-Acetyl-β-glucosaminidase	++
α-Mannosidase	++
α-Fucosidase	++

(+); Weak production: (++); Moderate production: (+++); Strong production

Table 3: Antibiotic sensitivity analysis of KCC-32

Name of Antibiotics	Concentration (µg)	KCC-32
Chloramphenicol (C)	50	S
Kanamycin (K)	30	S
Nitrofurantoin (NIT)	50	R
Tetracycline (TE)	100	S
Streptomycin (S)	25	S
Sulphafurazole (SF)	300	S
Colistin methane sulphonate (CL)	100	R
Dicloxacillin (D/C)	1	R
Ampicillin (AMP)	10	S
Amikacin (AK)	30	S
Gentamicin (GEN)	10	S
Cefoxitin (CX)	30	R
Cefalexin (CN)	30	S
Cefuroxime (CXM)	30	S
Co-Trimoxazole (COT)	25	R

>10mm: Susceptibility= S: R=Resistant

Table 4: Fermentative acid quantification in spent *Lactobacillus plantarum* KCC-32

Acid	Concentration (µg/mL)
Lactic	127.23±1.25
Acetic	28.3±1.04
Succinic	7.29±1.08

The values are expressed as mean ± SD of three replicates

plantarum KCC-32, fermented different carbohydrates. Further, KCC-32 secreted different kind of important enzymes like α-Galactosidase, β-Galactosidase, α-Glucosidase, and β-Glucosidase etc. β-Glucosidase breakdown the starch molecule and it converts the glycosides in to aglycones. The above conversion is a significant probiotic property because aglycones are easily absorbed by intestine (Ilavenil *et al*., 2016).

It is very crucial to check the antibiotic sensitivity of lactic acid bacterial strains before considering them for human and animal consumption (Fraqueza, 2015). In this experiment, *L. plantarum* KCC-32 showed sensitivity to most of the tested antibiotics, which confirm the nonpathogenic nature of KCC-32 strain. The fermentative products secreted by the lactic acid bacteria contain antimicrobial metabolites which act as competitive weapons between the microbes. These antimicrobial metabolite productions ignited significant interest among researchers for natural food preservation using lactic acid bacteria as bio perseverant (Coda *et al*., 2011). Subsequently, the estimation of fermentative acids resulted with a high amount

Table 5: Nutritive value of IRG silage according to inoculation of lactic acid bacteria

Treatment	CP[1)](%)	ADF[2)](%)	NDF[3)](%)	TDN[4)](%)
Control	9.11	33.29	52.28	62.60
L. plantarum; KCC-32	9.66	32.97	51.94	62.85

[1)] CP: Crude protein, [2)] ADF: Acid detergent fiber [3)] NDF: Neutral detergent fiber, [4)] TDN: Total digestible nutrient

Table 6: Changes of microbes on IRG silage according to inoculation of lactic acid bacteria

Treatment	LAB ($\times 10^7$ CFU[1)] /g)	Yeast ($\times 10^3$ CFU /g)	Fungi ($\times 10^3$ CFU /g)
Control	10.5[b]	14	0
L. plantarum KCC-32	24.3[a]	0	0

[1)] CFU: Colony per unit

a and b: Means with different letters within a column are significantly different at the 5% level

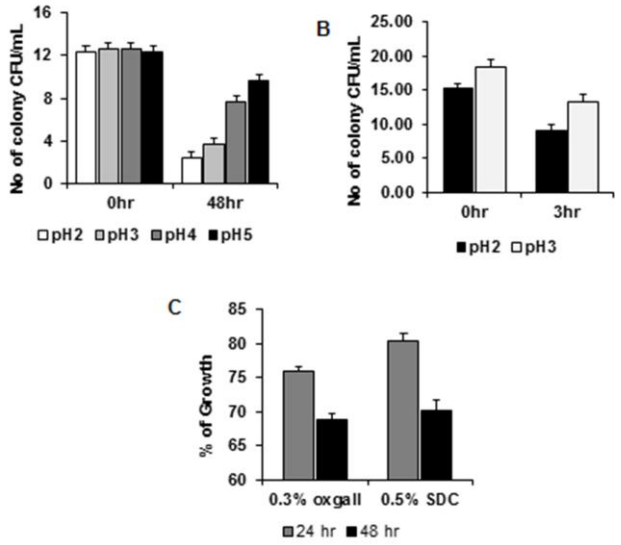

Fig. 1: Resistance of *L. plantarum* KCC-32 against low pH and bile salts

A, Resistance of KCC-32 against different pH; B, Resistance of KCC-32 against induced gastric juice; C, Resistance of KCC-32 against bile salts. The results are expressed as Mean of three replicates ± STD

of lactic acid production which connects the effective involvement of lactic acid in beneficial properties of KCC-32. Similarly, bile salt has been known to play a significant role in colonization and metabolic activity of microbes in the intestine (Leite *et al.*, 2015). Bile salts can act as an antimicrobial agent against intestinal microflora. Therefore, it is important to evaluate the bile salt tolerance of probiotic strains (Fontana *et al.*, 2013). KCC-32 showed resistant to both bile salts such as oxgall (0.3%) and sodium deoxycholate (0.5%), which agreement with the report of (Ilavenil *et al*., 2016).

Probiotic bacteria have many kinds of positive natural activities on the host that implies greater impact on host intestinal mucosal surface adherence, which

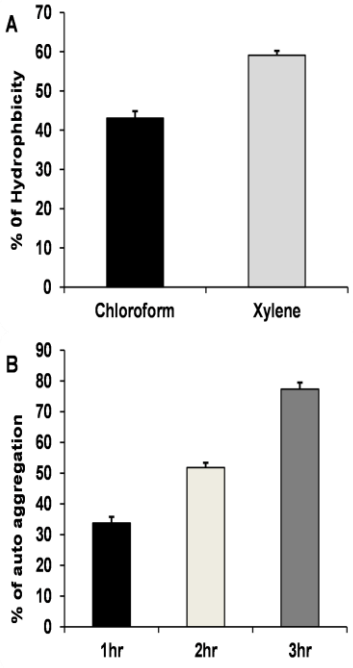

Fig. 2: Adhesion properties of *L.plantarum* KCC-32

A, Hydrophocity analysis of KCC-32; B, Autoaggregation analysis of KCC-32. The results are expressed as Mean of three replicates ± STD

useful for prolonged presence of probiotic strains in the gut. Mucus plays a significant role in the maintaining intestinal micro flora by promoting microbial binding, serving as a nutrient source, and matrix for bacterial proliferation (Ilavenil *et al.*, 2015). Conversely, mucus can inhibit pathogenic bacterial adhesion to the epithelium (Bautista-Gallego *et al.*, 2013). In this study, the hydrophobicity and auto aggregation experiments showed potential adhesion skills of *L. plantarum* KCC-32. Particularly, KCC-32 showed more hydrophobicity towards xylene when compared to chloroform and the rate of auto aggregation increased with increase in time. Hence, the results clearly confirmed the adhesion ability of KCC-32 towards intestinal environments.

In silage nutrient profile analysis the nutrient parameters such as ADF, CP, TDN and NDF play significant role in determining silage quality. Particularly, the increase in the CP level at animal feed indicates the increase in rumen protein degradation (Olmos Colmenero and Broderick, 2006). On the other hand addition of homo fermentative lactic acid bacteria in silage preparation could lead to decrease in protein breakdown (Merry *et al*., 2000). Accordingly, there is no significant changes were noted between control and

Table 7: Changes of pH and organic acids on IRG according to inoculation of lactic acid bacteria

Treatment	pH	Lactic acid (DM[1)]%)	Acetic acid (DM%)	Butyric acid (DM%)	Flieg′s score
Control	4.82[a]	3.10[a]	0.327	0.971[a]	58
L. plantarum; KCC-32	3.85[b]	4.891[b]	0.417	0.061[b]	88

[(1)] DM: Dry matter; a and b: Means with different letters within a column are significantly different at the 5% level

KCC-32 inoculated silage groups. Similarly the other nutrient parameters like ADF, NDF and TDN showed no significant changes in KCC-32 inoculated group when compared to control group. This result confirmed that the addition of KCC-32 in IRG silage increases the nutritive value of the silage and also agreed with (Avila *et al*., 2010).

The growth of undesirable microorganisms like fungi and yeast decrease the aerobic stability of the silage that increases the protein degradation and thereby increase the butyric acid production. The production of butyric acid in silage severely affects the flavor and quality of the silage (Jatkauskas and Vrotniakiene, 2004). Similarly no yeast and fungal growth were noted in KCC-32 inoculated group. Further, Addition of KCC-32 majorly increased the lactic acid production and then acetic acid production. The production of high amount of lactic acid confirmed the metabolic conversion of glucose and fructose in IRG silage by *L. plantarum* KCC-32 and agreement with (McDonald *et al*., 1991). Also, Most of the silages are affected by aerobic deterioration during warm climates (Ashbell *et al*., 2002). The aerobic yeasts are responsible for the aerobic deterioration of the silage and this could be eliminated by the addition of LAB inoculants (Danner *et al*., 2003). Accordingly, significant growth of *L. plantarum* increased the lactic acid and acetic acid production in KCC-32 inoculated group. The production of such organic acids automatically decreased the pH of the silage and thereby inhibited the growth of yeast colonies (Ilavenil *et al*., 2016). Hence, simultaneous production of lactic acid and acetic acid decreased the production of less significant end products like ammonia as well as volatile fatty acid that cause severe dry matter loss (Avila *et al*., 2012).

Conclusion

In this study the *L. plantarum* KCC-32 was isolated from fermented animal manure and characterized based on carbohydrate fermentation, physiological and 16S rRNA gene sequence analysis. The *in vitro* probiotic characteristic of KCC-32 confirmed its ability grow in gastro intestinal like condition. Further, inoculation of KCC-32 in IRG silage enhanced the quality and nutrient profile. Subsequently, KCC-32 produced high amount of lactic acid in MRS growth medium as well as silage preparation. The overall experimental result confirmed that KCC-32 could be used in quality animal feed preparation.

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