Open Access
Asian Australas. J. Anim. Sci.
Vol. 29, No. 2 : 250-256 February 2016
http://dx.doi.org/10.5713/ajas.15.0194
www.ajas.info
pISSN 1011-2367 eISSN 1976-5517

Effects of Graded Levels of Isomaltooligosaccharides on the Performance, Immune Function and Intestinal Status of Weaned Pigs

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ABSTRACT: The objective of this study was to investigate the effects of graded levels of isomaltooligosaccharides (IMO) on the performance, immune function and intestinal microflora and intestinal mucosal morphology of weaned pigs. In a 28-day experiment, one hundred eighty, twenty eight-day-old, crossbred (Duroc×Large White×Landrace), weaned pigs, with an initial body weight of 8.19±1.45 kg, were fed either an unsupplemented corn-soybean meal based diet or similar diets supplemented with 0.2%, 0.4%, 0.6%, or 0.8% IMO added at the expense of corn. Each treatment was replicated six times with six pigs (three barrows and three gilts) per pen. From day 0 to 14, weight gain was linearly increased ($p<0.05$), while gain:feed ($p<0.05$) was linearly improved and diarrhea rate ($p = 0.05$) linearly declined as the IMO level increased. On d 14, the level of the immunoglobulins IgA, IgM, and IgG in the serum of pigs were linearly increased ($p<0.05$) with increasing IMO supplementation. Interleukin-6 (IL-6) was linearly ($p<0.05$) and quadratically ($p<0.05$) decreased as IMO intake increased. From day 15 to 28, there was a trend for weight gain to be linearly increased, and IL-2 was linearly ($p<0.05$) increased as IMO supplementation increased on d 28. Over the entire experiment, weight gain was linearly increased ($p<0.05$), while gain:feed ($p<0.05$) was linearly improved and diarrhea rate ($p<0.05$) was linearly decreased as the IMO level increased. Supplementation with IMO had no effect on the intestinal microflora of pigs in the ileum and cecum of pigs, as well as the villus height and crypt depth in the ileum and jejunum ($p>0.05$). These results indicate that dietary inclusion of IMO increases weight gain, gain:feed and enhanced the immune status of pigs, and could be a valuable feed additive for use in weaned pigs, particularly during the period immediately after weaning. (**Key Words:** Immune Function, Intestinal Ststus, Isomaltooligosaccharides, Performance, Weaned Pigs)

INTRODUCTION

Isomaltooligosaccharides (IMO) have an α 1→6 glucosidic linkage in their molecular structure (Kohmoto et al., 1992), and are mainly derived from starch by an enzymatic transgalactosylation reaction (Hayashi et al., 1994; Vetere et al., 2000). Their composition is complex and the degree of polymerization ranges from di- to hexasaccharides (Kaneko et al., 1995b). They typically contain isomaltose, panose, isomaltotriose, and several other branched oligosaccharides made up of four or five glucose residues (Kaneko et al., 1995a).

Isomaltooligosaccharides are widely used in the food industry (Goffin et al., 2011), and have a wide spectrum of biological activities, such as improving the intestinal function in humans and animals (Chen et al., 2001; Ketabi et al., 2011; Yen et al., 2011). Furthermore, IMO are known for their potential to activate the immune system, thereby enhancing resistance to diseases and improving lipid metabolism (Wang et al., 2001; Mizubuchi et al., 2005; Li et al., 2009b).

Isomaltooligosaccharides have already been shown to have beneficial effects in a number of animal species including broilers (Zhang et al., 2003; Thitaram et al., 2005; Rehman et al., 2009), pigs (Li et al., 2009b; Li et al., 2010), shrimp (Li et al., 2009a; Zhang et al., 2011) and rats (Watanabe et al., 2002; Sung et al., 2004; Mizubuchi et al., 2005). In pigs, the beneficial effects appear to be mediated through enhancement of cell-mediated and humoral immunity of piglets (Li et al., 2010). However, the levels of IMO providing benefits for piglets have been inconsistent, so the optimum level of IMO for weaned pigs needs further

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Submitted Mar. 6, 2015; Revised Apr. 13, 2015; Accepted Jun. 3, 2015

research. The effects of supplementation with IMO in diets on intestinal microflora of weaned pigs and any potential risks in response to increasing inclusion dosages also needs to be studied. Therefore, the objective of the current experiment was to investigate the effects of graded levels of IMO in commercial diets on the performance, immune function, intestinal microflora and intestinal mucosal morphology in weaned pigs.

MATERIALS AND METHODS

The Institutional Animal Care and Use Committee of China Agricultural University (Beijing, China) approved the procedures used in this experiment. The IMO used in this study was provided by the Baolingbao Biology Company (Shandong, China) and its content of isomaltose, panose and isomaltotriose exceeded 45% (manufacturer's specifications).

Diets and experimental design

One hundred and eighty, twenty eight-day-old, crossbred (Duroc×Large White×Landrace) weaned pigs, with an initial body weight (BW) of 8.19±1.45 kg, were blocked by weight, sex, and ancestry and allotted to 1 of 5 dietary treatments in a 28-d experiment. The dietary treatments consisted of a corn-soybean meal based diet or similar diets (including roxarsone and olaquindox) supplemented with 0.2%, 0.4%, 0.6%, or 0.8% IMO added at the expense of corn (based on results of a preliminary experiment). During the first 14 days of this experiment, 2,000 ppm zinc oxide was added to all diets. The diets (Table 1) were formulated to meet or exceed nutrient requirements as suggested by the National Research Council (1998).

All pigs were housed indoors in 1.2 m×2.0 m concrete-floored pens with half of the pen area solid concrete and the remainder plastic slats. The pigs were provided *ad libitum* access to feed and water throughout the experimental period. The room temperature was maintained at 25°C to 27°C. Each treatment was replicated six times with six pigs (three barrows and three gilts) per pen.

The pigs were individually weighed on d 0, 14, and 28 of the experiment and feed consumption was measured per pen at the same time. These values were used to calculate weight gain, feed intake and gain:feed. All piglets were observed for evidence of scouring daily and the diarrhea rate was calculated. Fecal scores were monitored and quantified using a scale ranging from 0 to 3 as described by Marquardt et al. (1999), with 0 = normally shaped feces, 1 = soft feces, 2 = mild diarrhea, and 3 = severe diarrhea. A piglet with a score greater than 1 was regarded as having diarrhea. The diarrhea rate is expressed as the ratio between the number of pigs with diarrhea each day and the total number of pigs in each group (Zhao et al., 2015). On d 14 and 28, one pig was randomly chosen from each pen (3 males and 3 females for each treatment) and blood samples were collected from the vena cava cranialis using tubes without anticoagulant. Serum samples were separated by centrifugation at 1,342×*g* at 4°C for 10 min, and stored at –20°C until needed for analysis.

On d 28, the pigs which were blood sampled were slaughtered. The pigs were stunned by electric shock and then killed by exsanguination. The abdominal cavity was quickly opened to ensure a sterile operation and the ileum and cecum of each pig was isolated, and digesta samples were collected and stored at –20°C until analysis for intestinal microflora. Meanwhile, jejunum and ileum samples were flushed with normal saline to remove the

Table 1. Ingredient composition and nutrient content of basal diet (% as fed)[1,2]

Variable	%
Ingredient (%)	
Corn	58.52
Dehulled soybean meal[3]	16.50
Fish meal	4.00
Extruded soybean	10.00
Whey	4.00
Wheat bran	1.50
Soybean oil	1.50
Dicalcium phosphate	1.60
Limestone	0.60
DL-methionine (99%)	0.04
L-Lysine·HCl (78%)	0.34
Sodium chloride	0.35
Vitamin-mineral premix[4]	1.00
Mold inhibitor	0.05
Nutrient content[5]	
Metabolizable energy (MJ/kg)	14.08
Crude protein	19.21
Crude fiber	2.88
Ash	6.14
Calcium	0.82
Total phosphorus	0.65
Lysine	1.32

[1] Isomaltooligosaccharides were simply added to the control diet at concentrations of 0.2%, 0.4%, 0.6%, and 0.8% at the expense of corn.
[2] 2,000 ppm zinc oxide was added to the diets from day 0 to 14.
[3] Crude protein concentration of dehulled soybean meal is 47.60%.
[4] Provided per kg diet: vitamin A, 9,000 IU; vitamin D_3, 3,000 IU; vitamin E, 20 IU; vitamin K_3, 3.0 mg; thiamine, 1.5 mg; riboflavin, 4.0 mg; pyridoxine, 3.0 mg; cobalamin, 0.2 mg; biotin, 0.1 mg; pantothenic acid, 30 mg; nicotinic acid, 15 mg; folic acid, 0.75 mg; iron (from $FeSO_4 \cdot H_2O$), 75 mg; copper (from $CuSO_4$), 150 mg; zinc (from $ZnSO_4$), 75 mg; manganese (from $MnSO_4$), 60 mg; iodine (from KI), 0.35 mg; selenium (from Na_2SeO_3), 0.3 mg; roxarsone, 25 mg; olaquindox, 50 mg.
[5] All nutrient levels except metabolizable energy were chemically analyzed and the results are the means of duplicate determinations.

digesta and fixed by 10% formalin buffer for 48 h.

Chemical analysis

Calcium and total phosphorus in the diets were analyzed according to the methods of the Association of Official Analytical Chemists (2000), while crude protein was measured by the method of Thiex et al. (2002). For dietary lysine determination, feed samples were hydrolyzed with 6 *N* HCl for 24 h (AOAC, 2000). Ash was determined after ignition in a muffle furnace (Nabertherm, Bremen, Germany) at 500°C for 4 h. Crude fiber was determined according to the intermediate filtration method (ISO 6865:2000). Amino acids were chromatographically separated and quantified using a Model L-8900 Amino Acid Analyzer (Hitachi, Tokyo, Japan).

Samples were analyzed for serum immunoglobulins, including immunoglobulin A (IgA, which is an antibody that plays a critical role in mucosal immunity), immunoglobulin M (IgM, which is a basic antibody that is produced by B cells) and immunoglobulin G (IgG, which is synthesized and secreted by plasma B cells) and interleukins (ILs), including IL-2 (which is a cytokine signaling molecule in the immune system), IL-6 (which acts as an anti-inflammatory myokine) and IL-8 (which is a chemokine produced by macrophages and other cell types). All kits used in this trial were validated for pig serum (Kit numbers H0003, H0007, H0008, N0048, N0049 and N0050, Sino-uk Institute of Biological Technology, Beijing, China) following the standard procedures described by the manufacturer. Analyses were conducted using a Hitachi Auto-Analyzer (Hitachi 7020) and R-911 Automatic Radioimmunoassay Counter (China University of Technology Industrial Co., Hefei, China).

Microbiological analysis

Total aerobic bacteria, total anaerobic bacteria, *Escherichia coli* and *Lactobacillus* were analyzed by the Tablet Coated Colony Counting Method with three replicates. About 1 g of ileal and cecal digesta was diluted (1:10) in 9 mL aliquots of maximum recovery diluents (MRD, Oxoid, Basingstoke, UK), and 0.1 mL was chosen to spread onto selective agars. The medium for *Lactobacillus* was de Man, Rogosa and Sharp Agar (Oxoid), and incubation was carried out at 37°C overnight (18 to 24 h) in an atmosphere filled with 5% CO_2. The medium for *E. coli* and total aerobic bacteria were MacConkey and Brian Heart Infusion Agars (Oxoid), respectively and incubation was carried out at 37°C. The total anaerobic bacteria were isolated on Wilkins-Chalgren Anaerobe Agar (Oxoid) with 5% defibrinated sheep blood, and incubated 18 to 24 h at 37°C in an anaerobic tank (Mitsubishi Gas Chemical Co., Tokyo, Japan). Bacterial counts are expressed as colony-forming unit/g, and presented as $\log_{10}$-transformed data.

Intestinal histological analysis

Jejunum and ileum samples were dehydrated, paraffin sectioned and hematoxylin-eosin stained, then observed under the microscope to measure villus height and crypt depth with three sections examined for each of three replicates. Histological slices were examined with an Olympus BX51 microscope coupled with an Integrated Digital Imaging Analysis System (Olympus Co., Tokyo, Japan). Images were viewed (4×) to observe morphometric parameters of intestinal architecture. Villus height and crypt depths were measured manually. The villus height was denoted by the vertical distance from the crypt opening to the tip of the villus. The crypt depth was represented from the base of the crypt to the level of the crypt opening (Kik et al., 1990).

Statistical analysis

Analysis of variance was performed using pen means. Differences among treatments were examined using a one-way analysis of variance analysis version 8.0 (SAS Institute Inc., Cary, NC, USA). The linear and quadratic effects of IMO levels were assessed using orthogonal polynomial contrasts. A p-value of less than 0.05 was considered statistically significant, and less than 0.10 was considered a trend.

RESULTS

Performance

The effects of different levels of IMO on the performance of weaned pigs are presented in Table 2. From day 0 to 14, the daily gain ($p<0.05$) of pigs was linearly increased as the level of IMO supplementation increased, while gain: feed ($p<0.05$) was linearly improved. Diarrhea rate showed both a linear ($p = 0.05$) and quadratic ($p = 0.05$) decrease with increasing IMO supplementation. From day 15 to 28, there was a trend ($p = 0.07$) for increasing weight gain with IMO supplementation increased, but no effect on gain:feed or diarrhea rate of pigs. From day 0 to 28, weight gain was linearly increased ($p<0.05$), while gain:feed ($p<0.05$) was linearly improved and diarrhea rate ($p<0.05$) was linearly decreased as the IMO level increased.

Immune function

Table 3 presents the results of the analysis for serum immunoglobulins and ILs of pigs supplemented with different levels of IMO. On d 14, the level of the IgA, IgM and IgG in the serum of weaned pigs were linearly increased ($p<0.05$) with increasing IMO supplementation. IL-6 was linearly ($p<0.05$) and quadratically ($p<0.05$) decreased as IMO intake increased. On d 28, the level of

Table 2. Performance of 28-day old weaned pigs fed diets containing graded levels of isomaltooligosaccharides (d 0 to 28)

Item	Level of isomaltooligosaccharides (%)					SEM	p-value	
	0.0	0.2	0.4	0.6	0.8		Linear	Quadratic
0 to 14 d								
Daily gain (kg)	0.36	0.36	0.39	0.43	0.45	0.01	<0.01	0.45
Daily feed intake (kg)	0.57	0.62	0.64	0.61	0.66	0.02	0.31	0.79
Gain:feed	0.64	0.58	0.60	0.71	0.69	0.05	0.01	0.14
Diarrhea (%)[1]	8.93	2.50	2.86	4.28	2.14	1.53	0.05	0.05
15 to 28 d								
Daily gain (kg)	0.45	0.47	0.49	0.56	0.56	0.02	0.07	0.92
Daily feed intake (kg)	0.88	0.93	0.92	0.96	0.99	0.06	0.26	0.98
Gain:feed	0.51	0.51	0.53	0.60	0.57	0.11	0.11	0.99
Diarrhea (%)	3.21	4.64	3.57	1.78	1.43	1.50	0.21	0.48
0 to 28 d								
Daily gain (kg)	0.40	0.42	0.44	0.49	0.51	0.01	0.01	0.78
Daily feed intake (kg)	0.73	0.78	0.78	0.79	0.82	0.04	0.26	0.92
Gain:feed	0.56	0.54	0.56	0.64	0.62	0.06	0.02	0.55
Diarrhea (%)	6.07	3.57	3.21	3.04	1.79	1.13	0.02	0.56

SEM, standard error of the mean.
Values are the means of six replicates.
[1] Diarrhea, the ratio between the number of pigs with diarrhea each day and the total number of pigs in each group.

IL-2 was linearly ($p<0.05$) increased as IMO supplementation increased.

Intestinal microflora and intestinal mucosal morphology

The number of total aerobic bacteria, total anaerobic bacteria, *E. coli* and *Lactobacillus* in the ileum and cecum contents were unaffected by dietary treatments (Table 4). In addition, no significant difference was found in villus height and crypt depth in the ileum and jejunum, regardless of the dietary level of IMO (Table 5).

DISCUSSION

Isomaltooligosaccharides are oligosaccharides with interesting nutritional properties, and have been given considerable attention in the scientific field. Li et al. (2009b) indicated that dietary IMO (0, 25, 50, and 75 g/kg) fed to eight Large White castrated male pigs with an initial bodyweight of 25.6±1.1 kg for 8 days did not influence the total tract apparent digestibility of organic matter, crude protein and total ash, so that IMO had limited influence on the performance of growing pigs. However, the results of

Table 3. Serum biochemical measures of weaned pigs fed diets containing graded levels of isomaltooligosaccharides

Item	Level of isomaltooligosaccharides (%)					SEM	p-value	
	0.0	0.2	0.4	0.6	0.8		Linear	Quadratic
d 14								
Immunoglobulin A (g/L)	0.95	1.11	1.27	1.26	1.31	0.08	<0.01	0.21
Immunoglobulin M (g/L)	0.71	0.84	0.93	0.87	0.94	0.04	<0.01	0.13
Immunoglobulin G (g/L)	6.92	8.05	8.58	8.69	8.12	0.26	0.01	<0.01
Interleukin-2 (ng/mL)	4.87	5.10	5.58	5.44	4.67	0.37	0.96	0.11
Interleukin-6 (pg/mL)	216.77	162.64	169.11	148.68	156.17	9.96	<0.01	0.03
Interleukin-8 (ng/mL)	0.70	0.67	0.63	0.61	0.66	0.05	0.39	0.34
d 28								
Immunoglobulin A (g/L)	1.19	1.10	0.92	1.10	1.07	0.09	0.46	0.20
Immunoglobulin M (g/L)	0.90	0.90	0.98	0.83	0.83	0.05	0.29	0.28
Immunoglobulin G (g/L)	8.36	8.22	8.64	8.40	8.40	0.25	0.76	0.72
Interleukin-2 (ng/mL)	3.63	3.57	4.17	5.69	5.67	0.34	<0.01	0.51
Interleukin-6 (pg/mL)	143.39	136.56	141.66	158.06	147.24	11.84	0.47	0.94
Interleukin-8 (ng/mL)	0.38	0.34	0.34	0.53	0.33	0.03	0.51	0.49

SEM, standard error of the mean.
Values are the means of six replicates.

Table 4. Intestinal microflora (log_{10} CFU/g) of weaned pigs fed diets containing graded levels of isomaltooligosaccharides (d 28)

Item	Level of isomaltooligosaccharides (%)					SEM	p-value	
	0.0	0.2	0.4	0.6	0.8		Linear	Quadratic
Ileum								
Total aerobic bacteria	7.34	7.15	7.39	7.20	8.31	0.34	0.20	0.23
Total anaerobic bacteria	7.90	7.30	7.75	6.93	7.35	0.33	0.31	0.66
Lactobacillus	7.07	6.42	6.43	6.95	7.08	0.38	0.73	0.29
Escherichia coli	5.19	4.12	5.16	3.34	3.84	0.58	0.19	0.94
Cecum								
Total aerobic bacteria	7.67	7.44	7.54	7.26	6.62	0.34	0.12	0.49
Total anaerobic bacteria	7.77	7.95	8.26	7.75	6.66	0.41	0.20	0.12
Lactobacillus	7.73	7.83	8.17	7.76	7.47	0.21	0.54	0.17
Escherichia coli	3.96	3.87	3.96	3.86	3.60	0.58	0.75	0.85

SEM, standard error of the mean.
Values are the means of six replicates.

the present study showed that supplementation with 0.6% and 0.8% IMO significantly increased weight gain and gain:feed of weaned piglets. The main reason for this discrepancy might be that, the immune system is still developing in weaned pigs and therefore there is more likely to be a response to IMO in younger pigs than older pigs.

Studies concerning for effects of IMO supplementation for weaning piglets are scarce. Zhang et al. (2003) reported that supplementation with IMO enhanced performance only during the initial 3 wk, and the final BW of broilers consuming a diet with 0.3% IMO was somewhat greater at the end of 7 wk. However Thitaram et al. (2005) found that no differences existed in feed intake and feed conversion, although the weight of broilers fed 1% IMO showed a significant reduction.

Immunoglobulins, also known as antibodies, are large Y-shape proteins that mainly exist in the plasma and are used to identify and neutralize bacteria and viruses (Litman et al., 1993). ILs are a group of cytokines that were first found to be expressed by white blood cells and play an important role in the immune system (Brocker et al., 2010). They could activate and regulate immune cells, including mediating T and B cell activation, proliferation and differentiation and play an important role in the inflammatory response. The improvement in pig performance observed in the present experiment is most likely mediated by increases in IgA, IgM and IgG, since the increases of IgA, IgM and IgG indicate the activation of an immune reaction which might lead an improvement in immune status. Wang et al. (2012) reported that IMO had a positive effect on humoral and cell-mediated immunity for host animals. Mizubuchi et al. (2005) reported that the level of IgA in feces was increased when mice were fed a diet supplemented with 20% IMO (commercially available IMO, which contained 25.6% IMOs in the dry substance). This is in contrast to a study carried out previously which indicated that level of plasma IgA was not significantly affected in IMO treated rats (Sung et al., 2004).

In general, the main function of antibiotics is correlated with their capacity to eliminate a microorganism. However, toxins are released during the destruction of bacteria, which may, at least temporarily, deteriorate the condition of the microcirculation (AI-Banna et al., 2013). Thus, in this trial, antibiotics (including roxarsone and olaquindox) were added to the diets to enhance disease resistance, the function of IMO were not able to be completely expressed.

Mizubuchi et al. (2005) reported that IL-12 production was increased when mice were fed 20% IMO. However, there is little literature about IL changes when IMO are fed to pigs. The present study found that IL-6 was decreased in the earlier stage while IL-2 was increased in the later period

Table 5. Intestinal mucosal morphology of weaned pigs fed diets containing graded levels of isomaltooligosaccharides (d 28)

Item	Level of isomaltooligosaccharides (%)					SEM	p-value	
	0.0	0.2	0.4	0.6	0.8		Linear	Quadratic
Jejunum (μm)								
Villus height	280	294	308	306	290	10.41	0.41	0.20
Crypt depth	313	310	286	343	314	14.08	0.62	0.90
Ileum (μm)								
Villus height	341	315	371	347	316	16.28	0.98	0.39
Crypt depth	361	400	336	363	351	14.09	0.51	0.82

SEM, standard error of the mean.
Values are the means of six replicates.

with an increase in IMO supplementation. This is mainly because IL-6 is a factor involved in inflammation, while IL-2 is a kind of chemokine which could induce immune function.

Oligosaccharides (e.g. IMO) could inhibit enzymic reactions taking place in the upper part of the gastrointestinal tract, so that intestinal bacterial species might convert oligosaccharides into short-chain fatty acids by expressing specific hydrolases and gas by fermentation in the lower part (Ketabi et al., 2011). They could also promote some specific bacterial species (such as *Lactobacilli* and *Bifidobacteria*) selectively in the intestine, thus equilibrating the intestinal microflora (Delzenne, 2003). In the present study, the quantities of total aerobic bacteria, total anaerobic bacteria, *E. coli* and *Lactobacillus* in the ileum and cecum contents were unaffected by dietary treatment. This is similar to the finding that the total aerobes, *Lactobacillius*, and *E. coli* in the crop and cecum in broilers were not affected by the inclusion of IMO (Zhang et al., 2003). Wang et al. (2012) reported that increases in fecal *Lactobacilli* were observed when fermented milk supplemented with two probiotic strains and IMO were fed to mice.

Otherwise, IMO products differ substantially in composition, including the proportion of maltose and glucose, or the degree of polymerization, and such differences probably affect their functions on the intestinal microflora. In addition, the villus height and crypt depth of ileum and jejunum were not affected by the addition of IMO in this trial. The main reason might be that in the interaction of gut microflora and animal digestive enzymes (especially isomaltase), IMO were broken down and lost their expected stimulation on the substrate of the gut microflora. In conclusion, under these experimental conditions, a weaned piglet diet supplemented with 0.6% and 0.8% of isomaltooligosaccharide significantly improved piglet weight gain and gain:feed. Adding 0.2% to 0.8% IMO reduced the diarrhea rate of weaning piglets aged 0 to 14 days, and enhanced the immune status of pigs after weaning. However, the microflora in the ileum and cecum contents, and jejunum or ileum intestinal mucosa morphology were not affected by IMO addition.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

ACKNOWLEDGMENTS

This study was supported by the Ministry of Agriculture (Beijing, China), and we acknowledge the Baolingbao Biology Company for donation of the isomaltooligosaccharides used in this study.

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