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Abstract. Various antimicrobial agents were evaluated with the purpose of reducing the microbial fermentation in stored cattle waste and the resulting odor emissions. Duplicate sealed 2-L flasks with 500 ml waste slurry, with and without antimicrobial inhibitors, were used to measure the production of short-chain volatile fatty acids, lactate, and total fermentation gas over 27–30 days. A combination of chlorhexidine diacetate (2 mM), iodoacetate (2 mM), and α-pinene (3.8 mM) reduced gas production 80% (1000 ml to 200 ml) and total volatile fatty acid production 50% (145 mM to 72 mM). Pinene had little antimicrobial effect; rather, it served as an effective masking agent, giving the waste a less offensive odor. A combination of chlorhexidine diacetate and the deaminase inhibitor, diphenyliodonium chloride (1.3 mM) had a similar effect in reducing short-chain volatile fatty acid production (145 mM to 80 mM). It is concluded that a combination of antimicrobial agents may be useful in controlling odor emissions and conserving organic matter in livestock wastes, therefore providing a potentially more useful byproduct waste when used as plant fertilizer.

Current livestock production facilities generate some serious environmental concerns [13]. These concerns include large quantities of waste production in a small area resulting in surface and ground water pollution, atmospheric pollution, and the potential for transmission of pathogens. Odor, which is difficult to quantify or define, is one concern to which the general public gives the most attention.

Most of the offensive odor emitted from wastes results from an incomplete anaerobic degradation of the carbohydrate, protein, and lipid components [5]. This incomplete degradation results in the formation of short-chain volatile fatty acids, amines and other nitrogenous compounds, and sulfur-containing compounds, all of which are offensive. From a practical standpoint, complete anaerobic degradation of waste to methane has not been accepted, and the economics are suspect [7]. Aerobic treatment of livestock waste is not economically feasible and does little for conservation of nutrients. Solutions to managing the livestock waste should be simple, cost effective, and environmentally sound. Nutrient management should be a top priority.

Microbiologists have been trying to optimize the anaerobic fermentation in the rumen for 50 years [9]. It is well known that select plant components (condensed tannins, oils) inhibit rumen microorganisms. Therefore, one approach to control emissions from stored wastes might include adding natural antimicrobial agents and inhibitors to the waste to reduce the microbial activity. Inhibiting anaerobic microbial degradation pathways should result in less production and emission of offensive volatile organic compounds and gases from stored livestock wastes. The objectives of this laboratory study were to evaluate various antimicrobial agents for their ability to control the production of short-chain fatty acids, lactate, and gas production in stored beef cattle waste.

Materials and Methods

Waste slurry preparation. Fecal waste within 15 min of being excreted was randomly collected from a pen of 40 cattle fed a finishing 85% ground corn/15% forage diet. Urine was collected from catheterized cattle. Feces, urine, and distilled water in the ratio 50:35:15 were blended (Waring Inc., New Hartford, CT) for 1 min. Four replicate samples were obtained from this slurry and analyzed for various parameters and were considered as time 0. The waste slurry was divided into 500-ml aliquots, antimicrobial agents were added directly at the desired concentration without dissolving, slurry was blended 1 min to provide a homogeneous mixing of the antimicrobial agents, poured into a 2-L Erlenmeyer flask, which was sealed with a rubber stopper, and left stationary at ambient temperature (25°C). Treatments were in duplicate,

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and the contents in the flasks were gently swirled before being sampled at days 1, 2, 4, 7, 10, 14, 21, and 28 or as indicated.

Analytical methods. Gas volume and composition in the flasks were determined at times indicated in the figures. Headspace gas was measured by displacement of a water-lubricated glass piston in a 50-ml syringe when a 20-gauge needle was inserted through the stopper [6]. Methane and hydrogen were assayed with a gas chromatograph (model 3300; Varian Instruments) equipped with a thermal conductivity detector. The column was 0.2 cm by 305 cm stainless steel, which contained molecular sieve 5A (80/100 mesh; Restek, Bellefonte, PA). The column oven, injector, and detector were operated at 70°, 150°, and 200°C, respectively, and N_2 was the carrier gas. Purified methane and hydrogen were used as standards. Chromatographic data were integrated and concentrations calculated with a model SP 4290 integrator (Spectra-Physics, San Jose, CA).

A 15-ml waste sample was obtained from each flask after the flask was mixed by swirling it briefly. The sample was mixed with 15 ml of 0.5 M H_2SO_4, centrifuged at 2000 *g* for 20 min at 4°C, and stored at −20°C until analyzed [12]. L-Lactate concentrations were determined with a membrane-immobilized system involving lactate oxidase (EC 1.1.3.2; Model 27, Yellow Springs Instrument Co., Yellow Springs, OH). Short-chain volatile fatty acids (acetate, propionate, butyrate, valerate, isobutyrate, isovalerate) were determined in an aliquot from the original acidified sample. The acids were derivatized with tertiary butyldimethylsilyl according to the procedure of Richardson et al. [8]. A Hewlett-Packard 5890 GC (Wilmington, DE) with flame ionization detector, split injector (30:1), a 30 m × 0.25 mm DB5-30N capillary column (JW Scientific, Rancho Cordova, CA), and PC 1000 software (Thermal Separation Products, San Jose, CA) were used to analyze and calculate the acid concentrations. Sample injected was 1 µl and the column, injector, and detector temperatures were 60°, 250°, and 250°C, respectively; with hydrogen as a carrier gas.

Chemicals. All chemicals used in this study were purchased from Sigma Chemical Company (St. Louis, MO) or Aldrich (Milwaukee, WI).

Statistical analysis. Data were analyzed as a split-plot in time with the GLM procedure of Statistical Analysis System (SAS) [10]. Differences between means were tested with a linear model that included treatment and day as discrete effects. The model was treatment, flask nested within treatment, day, and treatment by day. Treatment means were tested with flask nested within treatment as the source of error. Day and treatment by day means were tested with the residual mean squares as the source of error. Least-square means are presented in the figures. Each mean represents duplicate samples from replicate treatments (n = 2).

Results

Several preliminary studies were conducted with a broad range of antimicrobial chemicals and metabolic inhibitors evaluated singly or in combinations to help focus on chemicals that may be effective in controlling the production of short-chain fatty acids, L-lactate, and gas production in stored cattle waste. These included 2-bromoethanesulfonic acid, anthraquinone, monensin, α-pinene, limonene, camphor, borneol, fenchol, eugenol, *p*-chloromercuribenzoate, diphenyliodonium chloride, chlorhexidine diacetate, iodoacetic acid, N,N′-dicyclohexylcarbodiimide, and methylglyoxal. Results from those chemicals which showed the highest inhibitory potential are presented here.

Data presented in Fig. 1 (A–D) indicated that chlorhexidine diacetate (2 mM) and iodoacetate (2 mM) added individually to the flasks exerted a metabolic effect. Noteworthy in this experiment was the fact that in all treatments, acetate, propionate and lactate increased very rapidly and leveled off at approximately day 10 (Fig. 1A, B, D). An exception to this was the treatment containing iodoacetate, which limited the propionate production to 17 mM compared with 22–24 mM for the other treatments (Fig. 1B). Sometime between days 10 and 15, a secondary fermentation occurred in all the treatments, except the one containing chlorhexidine diacetate, whereby acetate and lactate were converted to butyrate (Fig. 1A, C, D). In the chlorhexidine treatment, acetate and lactate remained at high levels and there was no increase in butyrate after day 2.

Various concentrations of chlorhexidine diacetate were evaluated, also in combination with iodoacetate and α-pinene, plus numerous other additions to this combination as indicated in Fig. 2 (A,B,C). The data in Fig. 2A indicate that chlorhexidine diacetate (2 mM) was the most effective ($P < 0.01$) in controlling the amount of fermentation gas in the treated flask. Approximately 100 ml of gas was produced compared with 1000 ml for the control flask. However, waste slurries containing 4 mM produced 600 ml of gas. The treatment in which the waste slurries were heated to 80°C for 1 h produced 2000 ml of gas. Approximately 20% of this gas was hydrogen, whereas the other treatments had only traces of hydrogen. The waste heated to 80°C for 1 h also contained butyrate at 95 mM (data not shown). Chlorhexidine diacetate, iodoacetate, and α-pinene, along with chlorhexidine diacetate and diphenyliodonium chloride, were the most effective ($P < 0.01$) treatments to control production of short-chain volatile fatty acids (Fig. 2B). Waste treated with chlorhexidine diacetate (4 mM) produced concentrations of volatile acids in excess of 300 mM, with acetate (140 mM) and propionate (120 mM) being the predominant acids; butyrate was less than 20 mM. However, 4 mM chlorhexidine diacetate was the most effective ($P < 0.01$) in controlling lactate production (Fig. 2C). The treatment containing the seven chemicals was less effective in controlling gas and short-chain volatile fatty acid production than the two treatments that contain chlorhexidine diacetate (2 mM) or chlorhexidine diacetate, iodoacetic acid, and pinene (Fig. 2 A,B). This suggests that the seven chemicals may interact with one another, reducing their potential ability to inhibit microbial metabolism.

Discussion

This study suggests that a combination of chlorhexidine diacetate (2 mM), iodoacetate (2 mM) and α-pinene (3.8

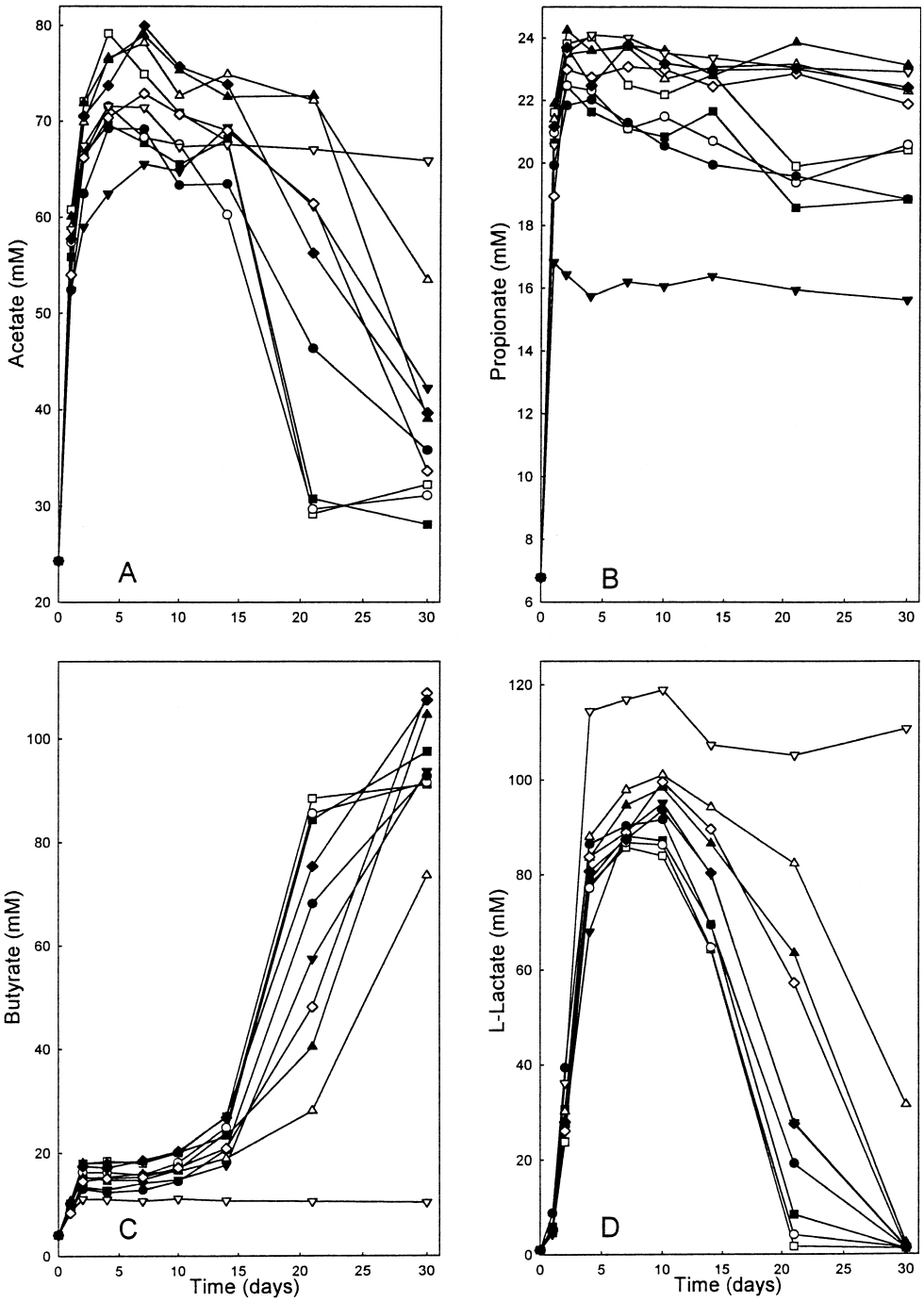

Fig. 1. Effect of various antimicrobial treatments on the production of acetate, propionate, butyrate, and L-lactate from stored beef cattle waste. Treatments include: □ control; ■ diphenyliodonium chloride 1.3 mM; ○ (+) camphor 3.3 mM; ● diphenyliodonium chloride 1.3 mM, and (+) camphor 3.3 mM; △ (−) α-pinene and (+) limonene 3.8 mM each; ▲ thymol 3.3 mM; ▽ chlorhexidine diacetate 2 mM; ▼ iodoacetate 2 mM; ◇ methylglyoxal 10 mM; ◆ N, N1-dicyclohexylcarbodiimide 2 mM. Treatment, day, and treatment by day interactions were significant ($P < 0.01$).

mM) can be used to effectively reduce the fermentation activity in stored beef cattle waste over a 27-day period. By inhibiting the production of fermentation gas and short-chain volatile fatty acids, especially acetate, propionate, and butyrate, less odor should be emitted from these wastes. This is supported by the study of Zahn et al. [14] in which they concluded that C2 through C9 organic acids from swine waste demonstrated the greatest potential for decreased air quality, since these compounds exhibited the highest trans-

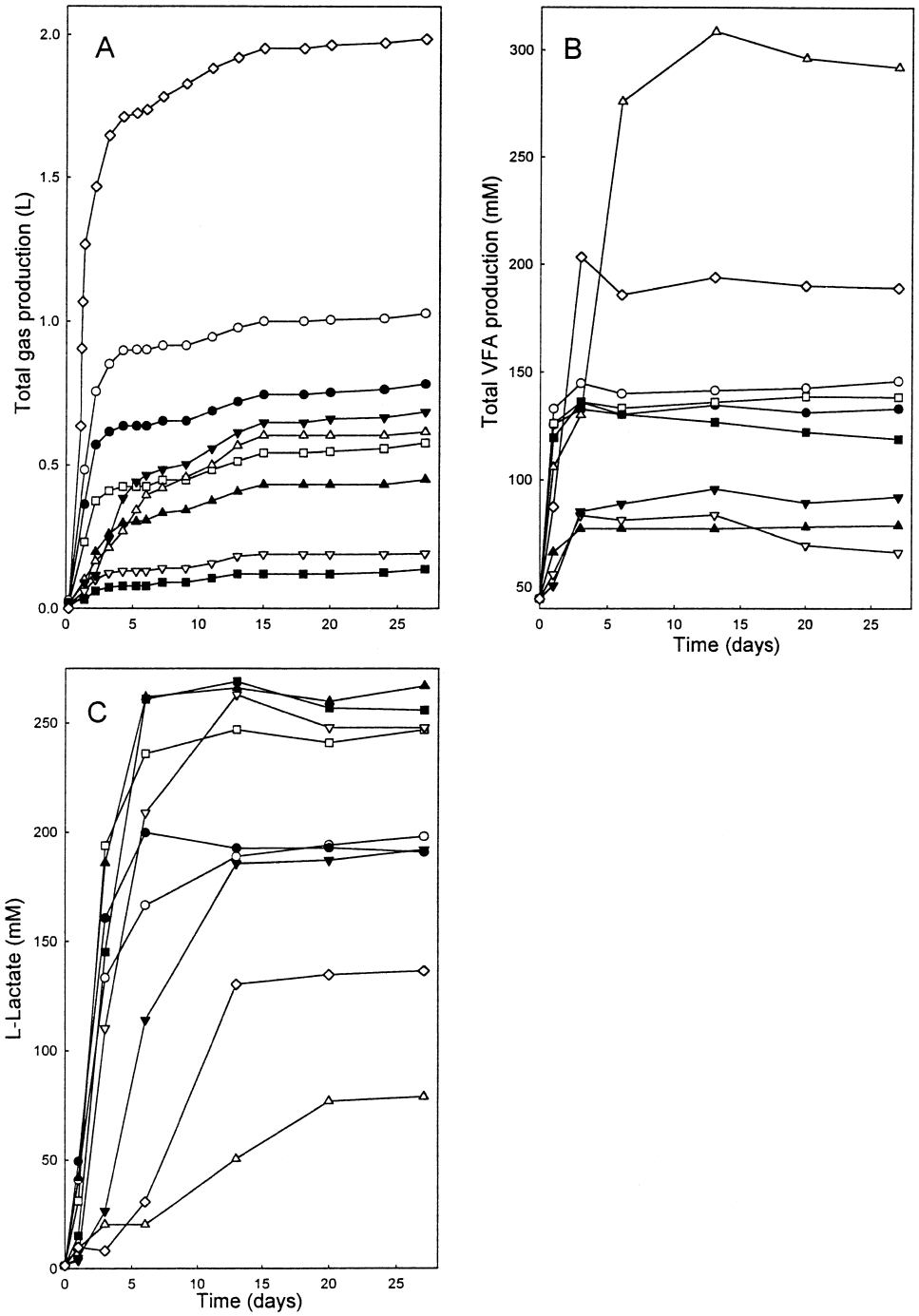

Fig. 2. Effect of various antimicrobial treatments on the production of total gas, total volatile fatty acids (VFA), and L-lactate from stored beef cattle waste. Treatments include: ○ control, ● chlorhexidine diacetate 0.5 mM; □ chlorhexidine diacetate 1.0 mM; ■ chlorhexidine diacetate 2 mM; △ chlorhexidine diacetate 4.0 mM; ▲ chlorhexidine diacetate 2 mM, and diphenyliodonium chloride 1.3 mM; ▽ chlorhexidine diacetate 2 mM, iodoacetic acid 2 mM, and (−) α-pinene 3.8 mM; ▲ chlorhexidine diacetate 2 mM, diphenyliodonium chloride 1.3 mM, (−) α-pinene and (+) limonene 3.8 mM each, iodoacetic acid 2 mM, methylglyoxal 10 mM, and N, N′-dicyclohexylcarbodiimide 2 mM; ◇ 80°C for 1 h. Treatment, day, and treatment by day interactions were significant ($P < 0.01$).

port coefficients and highest airborne concentrations. In our study, treatment of cattle waste with these three additives reduced the total volatile fatty acids from 145 mM to 72 mM, and gas production from 1000 ml to 200 ml (Fig. 2A, B). This is a reduction of 50% and 80% in the amount of volatile fatty acids and gas volume produced from the treated waste samples over a 27-day period.

Our data suggest that pinene, in combination with limonene, has limited antimicrobial activity in this treatment process (Fig. 1 A–D). This treatment decreased the conversion of acetate and lactate to butyrate (Fig. 1 A, C, D); however, this treatment had no effect on the initial production of acetate, propionate, and lactate when compared with the control (Fig. 1 A, B, D). A later study, with 7.5 mM α-pinene as a treatment, supported the conclusion that it has limited antimicrobial activity in this system (data not shown). The primary reason for including pinene in the treatment process is that it serves as an effective odor-masking agent, thereby giving the treated waste a less offensive characteristic odor.

Besides the treatment with chlorhexidine diacetate, iodoacetate, and pinene, a combination of chlorhexidine diacetate (2 mM) and diphenyliodonium chloride (1.3 mM) was also very effective in reducing the production of total volatile fatty acids (80 mM compared with 145 mM for control, Fig. 2 B). More fermentation gas (450 ml) was obtained from the chlorhexidine diacetate plus diphenyliodonium treatment than from the chlorhexidine, iodoacetic, and pinene treatment (200 ml). However, simply measuring volume of total fermentation gas without composition generally is a less reliable indicator of fermentation activity than quantifying fermentation acids. For instance, if a methanogenic fermentation is occurring, four moles of hydrogen would be reduced to one mole of methane (gas volume reduced fourfold). Essentially no methane was observed in our studies because of the high concentrations of acids in the waste. The final pH of the waste in all cases was between 4.0 and 5.0.

When the two most effective treatments listed above were repeated in another experiment with waste from different cattle, essentially the same results were obtained. When the combination of chlorhexidine diacetate (2 mM), iodoacetic acid (2 mM), diphenyliodonium chloride (1.3 mM), and pinene (3.8 mM) was evaluated, there was no additive inhibitory effect over the two earlier most effective treatments.

In these studies one of our treatments involved sterilizing the waste (15 psi, 121°C, 20 min). As expected, no fermentation gas or volatile fatty acids were initially produced. However, during our sampling procedure, which involved clean but non-sterile pipettes, we obviously contaminated these flask samples with a microorganism(s) that produced butyrate and hydrogen, because after 5 days butyrate increased from 8 mM to 24 mM and abundant gas was produced, which was approximately 20% hydrogen (data not shown). The fermentation occurring in these flasks, which were initially sterile, was very similar to the fermentation pattern we observed with the data presented in Fig. 2C, in which one of our treatments involved heating to 80°C for 1 h. Obviously we selected an organism such as *Clostridium butyricum*, a spore-forming organism, noted for its abundant butyrate and hydrogen production [3, 11]. This observation suggests that efforts to sterilize or pasteurize animal waste slurries will not be an effective long-term treatment to reduce the production of gas or odorous fermentation products. A residual chemical additive will be needed if these wastes are stored for extended periods such as 2–6 months, a common practice.

Chlorhexidine is a cationic bisbiguanide antimicrobial agent with a broad spectrum activity against bacteria and fungi. It is routinely used in mouth washes to control acid production by oral bacteria. Attia-Ismail et al. [1] evaluated the effectiveness of chlorhexidine in controlling lactate production by ruminal microorganisms. They found that it effectively reduced lactate production from glucose by mixed ruminal microorganisms; however, it also reduced acetate, propionate, butyrate, and total volatile fatty acids produced. This effect is detrimental to the ruminant animal because the volatile fatty acids are the primary energy source for the animal. In waste treatment systems, chlorhexidine, at higher concentrations than those used with rumen microorganisms, was marginally effective in controlling volatile fatty acid production, and only when used at 4 mM did it limit the production of lactate (Fig. 2C). However, when used in combination with iodoacetate, the two were very effective in inhibiting the overall fermentation in stored cattle waste. Iodoacetate is an inhibitor of glyceraldehyde-3-phosphate dehydrogenase, and chlorhexidine is thought to be an inhibitor of the phosphoenolpyruvate-dependent phosphotransferase system [4] and may affect other properties such as disrupting membrane integrity and function [1]. Diphenyliodonium chloride, a deaminase inhibitor [2], used in combination with chlorhexidine, was also an effective treatment to reduce the fermentation activity in cattle waste, but it did not give an additive effect to chlorhexidine and iodoacetate. The economics of these treatments are unknown; however, they will be pursued.

In conclusion, a combination of chlorhexidine diacetate, iodoacetate, and pinene should be an effective additive to stored livestock waste with the objective of inhibiting the microbial fermentation. This will, in turn, reduce odor emissions and minimize nutrient loss during waste storage. By conserving nutrients in the waste, the waste will have a higher composition of organic matter and will be a more useful product as a fertilizer for crop production.

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