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Effect of some fermentation conditions on antibacterial activity of extracted kefiran from kefir grains

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ABSTRACT

Antibacterial activities of kefir beverage are attributed to kefiran polysaccharide. The aim of this study was to investigate the effect of type of milk, fermentation time, temperature and stirring conditions on antibacterial activity of extracted kefiran from kefir grains. Kefir grains were added to full-fat and non-fat milk. Fermentation was carried out at 25°C and 37°C under stirred and non-stirred conditions. After 24, 48, 72 and 120 hours, the grains were separated from kefir extract and kefiran was extracted. The antibacterial activity of extracted kefiran was carried out by well method. Fermentation time had a significant effect on the inhibition zone of all tested bacteria. Milk type had significant effect on antibacterial activity of kefiran samples against tested bacteria except *S. dysenteriae*. Fermentation temperature had a significant effect on the inhibition zone of *E. coli* and *B. cereus*, but stirring conditions only had significant effect on inhibition zone of *B. cereus*. Considering all factors and their interactions, in order to extraction of kefiran with higher antibacterial activity is recommended, fermentation in full-fat milk. Also, to achieve higher antibacterial activity against *B. cereus, E. coli*, *S. dysenteriae,* and *S. aureus,* is recommended fermentation time for 24, 48, 48 and 120 hours, respectively. Fermentation at 37°C compared to 25°C and under stirred compared to non-stirred conditions did not create noticeable differences in the antibacterial activity of extracted kefiran from kefir grains. Fermentation conditions had significant effect on antibacterial activity of extracted kefiran from kefir grains.

1. Introduction

The history of the use of living microorganisms in food, especially lactic acid producing bacteria, in order to maintain and improve human health is very long. Probiotics are living microorganisms that, when consumed in sufficient quantities, have beneficial effects on human health (Fuller, 1989).

Probiotics may be a single strain, or a set of different microorganisms that kefir is an example of a probiotic that contains various types of bacteria and yeasts (Simova et al., 2002).

Kefir beverage is one of the oldest fermented milk products, a natural probiotic and alcohol-lactic beverage derived from fermented milk by kefir grains. Kefir is effective in promoting health, boosting the immune system, balancing blood pressure, treating gastrointestinal diseases and lowering serum cholesterol levels. It also

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has antibacterial, antifungal and anti-tumor activities (Farnworth, 2005).

Kefir grains, jelly-like grains, resemble small cauliflower grains. They are irregular in shape and white to yellow and have a slim firm texture (Farnworth, 2005).

The reproductive capacity of kefir grains is significantly affected by growth conditions. Under favorable conditions, after numerous passages in milk, they recover their normal appearance, physiological functions and technological properties (Pop et al., 2014).

The grains contain a group of specific microorganisms that coexist in a complex symbiotic relationship and includes yeast species, lactic acid bacteria (Lactobacillus and Lactococcus) and acetic acid bacteria (Garbers et al., 2004).

The microorganisms in kefir grains are surrounded by a protein matrix and polysaccharide called kefiran. Kefiran is a branch glucogalactane of water-soluble consisting of identical amounts of D-galactose and D-glucose. Kefiran production is mainly done by the species *Lactobacillus kefiranofacience* and *Lactobacillus kefir*. The antibacterial, antifungal and antitumor activities of Kefir beverage are attributed to Kefiran. This polysaccharide because of its health benefits, has significant potential as a food gum in the food industry, in the production of new packaging materials or as a food enhancer due to its health benefits (Piermaria et al., 2009; Prado et al., 2015). Kefiran production can be dramatically increased by controlling the conditions of cultivation and modification of the intermediate composition, also increasing kefir grains and producing kefiran stimulated by adding mineral resources (Frengova et al., 2002; Zajsek and Gorsek., 2011).

The aim of this study was to investigate the effect of some fermentation conditions including type of milk, fermentation time, temperature and stirring conditions on antibacterial activity of extracted kefiran from kefir grains against four gastrointestinal pathogenic bacteria including *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus* and *Shigella dysenteriae*.

2. Materials and Methods

2.1. The process of fermentation of kefir grains

The method presented by Pop et al., 2014; Zajsek and Gorsek., 2011 was used to perform the fermentation process. Briefly, Kefir grains were purchased from a local supermarket and were recovered by sequential subcultures in milk for 4 days at 25°C. The milk was replaced every 24 hours. After resuscitation, kefir grains were washed with sterile distilled water and 5 g of them were inoculated separately to 50 ml of full-fat and non-fat milk (Skim milk). The samples were incubated at 25°C and 37°C under stirred and non-stirred conditions. At 24, 48, 72, and 120 h intervals, kefir grains were isolated from the fermentation product. Separated kefir grains were used to extraction of kefiran.

2.2. Extraction of kefiran

The method presented by Zajsek and Gorsek., 2011 and Rimada et al., 2003 was used to perform the extraction of kefiran. Briefly, 50 ml of boiling water was added to kefir grains and placed on a magnet heater for 3 hours to dissolvation of the kefiran polysaccharides and inactivation of the hydrolyzing enzymes. Upon reaching ambient temperature, 8.5 ml of 80% trichloroacetic acid was added to each of the treatments, and after overnight the samples were centrifuged at 4°C for 20 min at 10,000 rpm. The same volume of supernatant was added ethanol 97% and in order to precipitation of kefiran stored for overnight at -20°C. The solution was centrifuged at 4°C for 20 min at 10,000 rpm and the precipitate was washed with boiling water. Washing procedure of the precipitate was repeated three times in boiling water, and the final precipitate was dried at 42°C for 48 hours. Finally, dry weight of kefiran was measured.

2.3. Preparation of Bacterial Strains

The strains of the tested bacteria were two gram-negative bacteria of *Escherichia coli* (PTCC 1338) and *Shigella dysenteriae* (PTCC 1188), and two gram-positive bacteria of *Staphylococcus aureus* (PTCC 1112) and *Bacillus cereus* (PTCC 1154). They were prepared from the Iranian Research Organization for Science and Technology (IROST) in a lyophilized form. Then, they were recovered in BHI (Brain Heart Infusion) medium (Merck) for 24 h at 37°C in the microbiology laboratory of Islamic Azad University of Azadshahr branch according to the method of Weinstein et al. 2018. The 24-hour culture of each bacterium was inoculated into Nutrient Broth culture

medium (Merck) and it was incubated at 37°C to obtain turbidity equal to 0.5 McFarland $=1.5\times10^8$ CFU/ml.

2.4. Evaluation of antibacterial activity by well method

Antibacterial activity of extracted kefiran samples from kefir grains was carried out based on agar diffusion and using by well method. For this purpose, the method presented by Weinstein et al. 2018 was used. Briefly, a bacterial suspension equivalent to 0.5 McFarland (1.5×10^8 CFU/ml) was prepared from all bacterial strains and a uniform culture was prepared from this suspension on the surface of the Mueller Hinton Agar medium (Merck). Then, wells with a diameter of 8 mm were created by using a cork borer. 100 µL of extracted kefiran samples (300 mg/ml) were poured into wells and incubated at 37°C for 24-48 hours. Following that, the sensitivity and resistance of the each tested bacteria was determined by measuring the inhibition zone diameter around the wells (Weinstein et al., 2018).

2.5. Determination of MIC (Minimum Inhibitory Concentration) and MBC (Minimum Bactericidal Concentration) of extracted kefiran samples

Determination of MIC of extracted kefiran samples were carried out based on turbidimetric assay and by microdilution method using 96-well ELISA microplates. For this purpose, serial dilutions of extracted kefiran samples were prepared in Nutrient Broth (Merck) then to each of these wells was added suspensions equivalent to 5×10^5 CFU/ml from each of the bacteria and incubated for 24 h at 37°C. There were also control tubes containing of serial dilutions of extracted kefiran samples were prepared in Nutrient broth (without bacterial suspension) as negative controls and bacterial suspension of 5×10^5 CFU/ml (without extracted kefiran) as positive controls. The results after 24 h of incubation for microbial turbidity of visible were recorded. The last dilution (lowest concentration) in which microbial turbidity was not observed, as the MIC was considered. For the determination of MBC, from the tube that contained extracted kefiran samples concentrations higher than the MIC were cultured onto the Nutrient agar medium. The MBC was defined as the lowest concentration that allowed no visible growth on the agar (Weinstein et al., 2018).

2. 6. Statistical design and data analysis

The study was conducted in factorial template with completely randomized design with three replications. Factors included two levels of type of milk (Full-fat and Non-fat milk (Skim milk)), two levels of stirring conditions (Stirred and Non-stirred), two temperature levels (25°C and 37°C) and four time levels (24, 48, 72 and 120 hours) that are listed in Table 1. The responses include the inhibition zone diameter of four *E. coli*, *S. aureus*, *B. cereus* and *S. dysenteriae* in the presence of extracted kefiran extraction (mm). Design-Expert software (Version 12.0.3.0) was used to data analysis and draw charts.

Table1. Factors investigated in the study

No	Factor	Name	Units	Type	Minimum	Maximum	evels
1	A	Temperature	°C	Categoric	25	37	2
2	B	Stirred/Non stirred		Categoric	Stirred	Non-stirred	2
3	C	Time	Hour	Categoric	24	120	4
4	D	Type of Milk		Categoric	Full-Fat Milk	Non-Fat Milk	2

3. Results and Discussion

In Table 2 presents the mean of the inhibition zone diameter of the tested bacteria included 32 treatments, related to the effect of different fermentation conditions ie 2 levels of milk type (Full-fat and non-fat milk), 2 levels of stirring conditions (stirred and non-stirred), Two temperature levels (25 and 37°C) and four time levels (24, 48, 72, and 120 h) on antibacterial activity of extracted kefiran samples (The results of antibacterial activity are based on the inhibition zone diameter and the mean of three replicates).

3.1. Results of analysis of variance the impact of different fermentation conditions on the inhibition zone of the tested bacteria

Analysis of variance of mean of inhibition zone diameter for tested bacteria showed that, fermentation time had a significant effect on the mean of inhibition zone diameter of all tested bacteria against of extracted kefiran samples. Also type of milk had significant effect on antibacterial activity of extracted kefiran samples against tested bacteria except *S. dysenteriae*. Fermentation temperature had a significant effect on the inhibition zone diameter of *E. coli* and *B. cereus*, but stirring conditions only had significant effect on inhibition zone diameter of *B. cereus* against extracted kefiran samples (Table 3).

In the study of the interaction effects of variables, the interactions of temperature with fermentation time, temperature with stirring conditions, temperature with milk type, stirring conditions with fermentation time and fermentation time with milk type had significant effect on the mean of inhibition zone of all tested bacteria against extracted kefiran samples. Only interaction effects of stirring conditions with milk type had not significant effect on antibacterial activity of extracted kefiran samples against *S. dysenteriae* and *B. cereus*.

The interaction of three factors of temperature with stirring conditions and fermentation time and the interaction of three factors of temperature with time and milk type had significant effect on antibacterial activity of extracted kefiran samples against all tested bacteria. But the interaction of three factors temperature with stirring conditions and milk type had significant effect on the mean of inhibition zone of *E. coli* and *B. cereus* and the interaction of stirring conditions with fermentation time and milk type had significant effect on the antibacterial activity of kefir samples against *S. dysenteriae* and *B. cereus* (Table 3).

Finally, the interaction of four factors of temperature, stirring conditions, fermentation time and milk type had significant effect on the mean of inhibition zone diameter of the tested bacteria except for *S. aureus* (Table 3).

Given that the difference between Predicted R^2 and Adjusted R^2 values is less than 0.2 and Adeq Precision values greater than 4, indicates the model's desirability and them navigation (Table 3).

3.2. Effect of fermentation time on antibacterial activity of extracted kefiran samples

Analysis of variance showed that fermentation time had a significant effect on the antibacterial activity of extracted kefiran samples against all tested bacteria (Table 3).

Although the highest inhibition zone of *S. dysenteriae* and *S. aureus* was observed in extracted kefiran samples from fermented kefir grains for 48 hours with mean of inhibition zone of 22 and 21.66 mm respectively, and the highest inhibition zone of *B. cereus* and *E. coli* in fermented samples for 24 hours with mean of inhibition zone of 25.33 and 17.33 mm respectively (Table 2), but considering all factors and their interactions, extracted kefiran samples from fermented kefir grains for 48 hours had the highest antibacterial activity against *E. coli* and *S. dysenteriae* and extracted kefiran samples from fermented kefir grains for 24 and 120 hours showed the highest antibacterial activity against *B. cereus* and *S. aureus* (Fig. 1-8).

There are various reports of the effect of fermentation time on the antibacterial activity of kefir. The antibacterial activity of kefir fermented beverages is attributed to kefiran polysaccharide (Frengova et al., 2002; Zajsek and Gorsek., 2011). Therefore, increasing and decreasing antibacterial activity of kefir samples may be related to the incidence or absence of antimicrobial nature of this compound at different fermentation times. For example, weschenfelder and colleagues reported that the antibacterial activity of kefir samples against *S. aureus* and *E. coli* it starts after 24 hours. In this study, maximum antibacterial activity against *E. coli* was observed after 48 and 72 hours' fermentation (Weschenfelder et al., 2018).

Kim et al. (2016) in the study of antibacterial activity of four kefir samples at fermentation times of 24, 36, 48 and 72 hours against 8 gastrointestinal pathogens have reported the highest antibacterial activity in all fermented samples for 36 and 48 hours (Kim et al., 2016). On the other hand, Dias et al. (2012) reported the survival of *E. coli* and *S. aureus* in kefir for 72 hours (Dias et al., 2012).

Increased antibacterial activity of kefir samples with increasing fermentation time has been reported in several studies (Silva et al., 2009; Rahimzadeh et al., 2012 and 2015).

Due to the importance of the role of kefiran in the antibacterial activity of kefir fermented beverages, numerous studies have reported on the best time to extraction of kefiran from kefir grains. Some studies have reported 24-hour fermentation times (Zajsek and Gorsek, 2011; Pop et al., 2014). On the other hand, fermentation time of 120 hours has been reported for maximum kefiran extraction (Ismaiel et al., 2011). Some studies reported longer times, such as 7 days (Maeda et al., 2003) and 10 days (Taniguchi et al., 2001) to produce the highest amount of kefiran by *Lactobacillus kefiranofaciens*.

As indicated in the present results, the highest antibacterial activity of extracted kefiran samples against *B. cereus*, *E. coli*, *S. dysenteriae* and *S. aureus* were from fermented kefir grains for 24, 48, 48 and 120 hours, respectively. There are varied reports of the best fermentation time for the highest antibacterial activity of kefir samples and the best fermentation time for extraction the highest kefiran (as mentioned above), justifies the diversity and difference of the best time for the highest antibacterial activity in the extracted kefiran samples in this study. This may be related to the effect of other compounds produced at different fermentation times of kefir on the structure and function of kefiran composition. Because kefir grains produce different products at different fermentation times.

Concerning the antibacterial activity of extracted kefiran samples from kefir grains against the tested gram negative bacteria, we observed a decrease in antibacterial activity over long periods of 72 and 120 hours. The rational justification for this decrease in antibacterial activity over long periods of time may be the hydrolysis of polysaccharide in its monomers by enzymatic degradation (Pop et al., 2014).

It has also been observed in the study of Pop et al. That the mass of kefir grains increased after 24 h but decreased at 48 and 72 h at 25 ° C. Reduced nutrient or excess acidity for biological activities due to lactic acid production may be the reason degradation (Pop et al., 2014).

Of course, there are also reports of lower levels of exopolysaccharides even during cold storage (Kok-Tas et al., 2013; Ramachandran and shah, 2009).

3.3. Effect of stirring conditions on antibacterial activity of extracted kefiran samples

The highest the mean of inhibition zone against extracted kefiran samples, was observed for *S. aureus*, *B. cereus* and *S. dysenteriae* under non-stirred conditions whereas for *E. coli* and *S. dysenteriae* under stirred conditions (Table 2). Also, considering all the factors and their interactions, no noticeable difference was observed between the antibacterial activity of extracted kefiran samples under stirred and non-stirred conditions (Fig. 1–8). Of course, the results of analysis of variance also showed that stirring conditions did not have a significant effect on the mean of inhibition zone diameter of the tested bacteria except *B. cereus* (Table 3).

Considering the essential role of kefiran polysaccharide in the antibacterial activity of fermented milk by kefir grains (Frengova et al., 2002; Zajsek and Gorsek., 2011), studies have been carried out on the role of stirring conditions in the extraction rate of this polysaccharide from kefir grains that have reported a variety of results. There is research that has reported a higher rate of extracted kefiran in non-stirred conditions (Ismaiel et al., 2011). Other studies, however, have pointed to the role of stirring in increasing kefiran production (Pop et al., 2014; Zajsek and Gorsek, 2011).

The researchers believe that stirring with increasing of mixing preserves homogeneous fermentation conditions as well as enhances nutrient and air mass transfer, which justifies more kefiran extraction in stirred media than non-stirred media (Zajsek and Gorsek., 2011).

No difference in the antibacterial activity of extracted kefiran samples from both stirred and non-stirred conditions in the present study may be due to the fact that stirring conditions do not affect the nature of the kefiran compound and its biological activities.

3.4. Effect of milk type on antibacterial activity of extracted kefiran samples

The results of analysis of variance showed that type of milk had significant effect on the

mean of inhibition zone diameter of the tested bacteria except *S. dysenteriae* (Table 3).

The highest the mean of inhibition zone against extracted kefiran samples against tested bacteria, was observed in fermented samples in full-fat milk (Table 2). Considering all the factors and their interactions, also extracted kefiran samples from fermented kefir grains in full-fat milk were showed higher antibacterial activity against tested bacteria (Fig. 1–8).

Various studies have pointed to the positive role of full-fat milk, raw milk and pasteurized milk in comparison with skimmed milk in the extraction of kefiran from kefir grains and the antibacterial activity of kefir extract.

Florence et al (2012) suggested raw milk as a suitable substrate for production of fermented milks (Florence et al., 2012). Zajšek and Goršek also proposed full-fat cows' milk as the best environment for the production of kefiran from kefir grains (Zajsek and Gorsek, 2011).

Weschenfelder et al. Reported more antibacterial activity of prepared kefir samples in pasteurized milk compared to prepared kefir samples in from skimmed milk (Weschenfelder et al., 2018). This may be related to the dependence of microorganisms present in kefir grains to substrates with high protein and fat content in order to producing compounds of with antimicrobial nature such as kefiran. The antibacterial activities of kefir beverage are attributed to kefiran and the production of kefiran can be dramatically increased by controlling the culture conditions (Frengova et al., 2002; Zajsek and Gorsek., 2011). More antibacterial activity of produced kefir samples in pasteurized milk, which has more fat than skimmed milk, indicates the production of more kefiran in these samples.

More antibacterial activity of extracted kefiran samples from fermented kefir grains in full-fat milk compared to non-fat milk in the present study, confirms the dependence of kefir grains growth on the presence and amount of substrates required for the growth of microorganisms present in kefir grains including protein and lactose.

3.5. Effect of fermentation temperature on antibacterial activity of extracted kefiran samples

Although the highest inhibition zone diameter of tested bacteria, with the exception of *E. coli*, was observed against extracted kefiran samples from fermented kefir grains at 25°C (Table 2), but considering all factors and their interactions, no noticeable difference was observed between the antibacterial activity of extracted kefiran samples against *S. aureus*, *S. dysenteriae* and *B. cereus* at fermentation temperatures of 25 and 37°C, and only the mean of inhibition zone of *E. coli* against extracted kefiran samples from fermented kefir grains at 37°C was more than 25°C (Fig. 1-8). On the other hand, the results of analysis of variance also showed that the fermentation temperature had only a significant effect on the mean of inhibition zone diameter of *E. coli* ($P < 0.0001$) and to some extent *B. cereus* ($P < 0.0491$) (Table 3).

These results indicate that the fermentation temperature range of 25 and 37°C could not make a change in the nature of the antimicrobial compounds of the kefir (kefiran) samples and that the kefiran polysaccharide is not very sensitive to temperature changes in the range of 25 to 37°C. Of course, this is different for *E. coli*. Because the analyzes showed that the antibacterial activity of extracted kefiran samples from fermented kefir grains at 37°C had more inhibitory effects against *E. coli*.

Since traditional kefir beverages are prepared at 25°C for 18-24 hours (Farnworth and Mainville, 2008). Several studies have indicated the highest rate of kefiran extraction from kefir grains at 25°C (Pop et al., 2014; Zajsek and Gorsek, 2011). Maximum production of kefiran at 25°C is due to the fact that microorganisms protect themselves against environmental influences by increasing their kefiran production (Zajsek and Gorsek., 2011).

Studies have also reported the highest rate of kefiran extraction from kefir grains at 30°C (Ismaiel et al., 2011).

Numerous studies have reported temperature of 30°C as the best temperature for the highest production of kefiran by *Lactobacillus kefiranofaciens* (Yokoi and Watanabe, 1992; Tanaguchi et al., 2001; Yeesang et al., 2007). Reduce the extraction rate of kefiran at higher temperatures can be attributed to the dissolution of this exopolysaccharide at high temperatures (Rimada and Abraham., 2001).

Table 2. The mean of the inhibition zone diameter of the tested bacteria against extracted kefiran samples in different fermentation conditions

Treatment	Temperature (°C)	Kind of milk*	Stirred/Non Stirred**	Time (h)	Inhibition zone of bacteria (mm)***				Desirability
					S. aureus	*B. cereus*	*S. dysenteriae*	*E. coli*	
1	25	NFM	NS	24	9.667	11.333	10.000	9.667	**1.000**
2	25	NFM	NS	48	11.667	10.000	17.000	11.667	**1.000**
3	25	NFM	NS	72	10.333	10.667	11.333	8.000	**1.000**
4	25	NFM	NS	120	13.333	24.667	18.000	11.333	**1.000**
5	25	NFM	S	24	8.000	8.000	8.000	8.000	**1.000**
6	25	NFM	S	48	9.000	11.333	12.667	8.000	**1.000**
7	25	NFM	S	72	10.333	11.000	11.333	9.667	**1.000**
8	25	NFM	S	120	18.333	19.000	20.333	14.000	**1.000**
9	25	FFM	NS	24	15.667	25.333	14.667	12.667	**1.000**
10	25	FFM	NS	48	21.667	22.333	22.000	15.667	**1.000**
11	25	FFM	NS	72	10.333	12.333	11.667	8.667	**1.000**
12	25	FFM	NS	120	12.667	11.000	12.333	9.333	**1.000**
13	25	FFM	S	24	12.000	22.333	16.333	11.000	**1.000**
14	25	FFM	S	48	15.667	20.333	22.000	15.667	**1.000**
15	25	FFM	S	72	11.333	12.333	12.333	9.000	**1.000**
16	25	FFM	S	120	11.667	11.333	10.000	9.333	**1.000**
17	37	NFM	NS	24	12.667	10.667	17.667	10.667	**1.000**
18	37	NFM	NS	48	12.333	17.667	17.333	15.333	**1.000**
19	37	NFM	NS	72	12.667	14.000	12.333	12.667	**1.000**
20	37	NFM	NS	120	11.333	13.000	11.333	9.667	**1.000**
21	37	NFM	S	24	14.000	21.333	21.000	11.667	**1.000**
22	37	NFM	S	48	12.000	13.667	11.333	11.667	**1.000**
23	37	NFM	S	72	13.667	13.333	12.000	12.333	**1.000**
24	37	NFM	S	120	14.333	24.000	16.000	12.000	**1.000**
25	37	FFM	NS	24	14.333	23.333	17.000	11.667	**1.000**
26	37	FFM	NS	48	11.000	13.333	12.667	13.000	**1.000**
27	37	FFM	NS	72	12.667	12.667	12.000	11.667	**1.000**
28	37	FFM	NS	120	10.667	10.667	11.667	8.000	**1.000**
29	37	FFM	S	24	15.667	20.000	17.333	17.333	**1.000**
30	37	FFM	S	48	9.667	11.333	10.667	8.000	**1.000**
31	37	FFM	S	72	12.333	12.333	11.667	11.333	**1.000**
32	37	FFM	S	120	14.000	21.667	19.333	16.667	**1.000**

Table 3. p-value and other parameters extracted from analysis of variance table

	E. coli	*S. dysenteriae*	*S. aureus*	*B. cereus*
Source		**p-value**		
Model	< 0.0001	< 0.0001	< 0.0001	**< 0.0001**
A: Temperature	< 0.0001	0.7640	0.7180	**0.0491**
B: Stir (Stirred/Non Stirred)	0.1067	0.4537	0.8284	**0.0358**
C: Time (Hour)	< 0.0001	< 0.0001	0.0020	**< 0.0001**
D: Type of milk (Full fat/Non fat)	0.0010	0.1796	0.0003	**< 0.0001**
AB	0.0050	0.0127	0.0005	**< 0.0001**
AC	< 0.0001	< 0.0001	< 0.0001	**< 0.0001**
AD	0.0133	< 0.0001	< 0.0001	**< 0.0001**
BC	< 0.0001	< 0.0001	< 0.0001	**< 0.0001**
BD	0.0133	0.0751	0.0092	**0.0664**
CD	< 0.0001	< 0.0001	< 0.0001	**< 0.0001**
ABC	< 0.0001	< 0.0001	0.0405	**< 0.0001**
ABD	0.0082	1.0000	0.0744	**0.0041**
ACD	< 0.0001	< 0.0001	< 0.0001	**< 0.0001**
BCD	0.1158	0.0128	0.3673	**< 0.0001**
ABCD	< 0.0001	0.0004	0.0781	**< 0.0001**
R	0.8903	0.9222	0.8470	**0.9491**
Adjusted R	0.8372	0.8845	0.7728	**0.9245**
Predicted R	0.7532	0.8249	0.6557	**0.8855**
Adeq Precision	14.3993	17.9089	16.8260	**20.3472**

*** P ≤ 0.001, ** P ≤ 0.01, * P ≤ 0.05

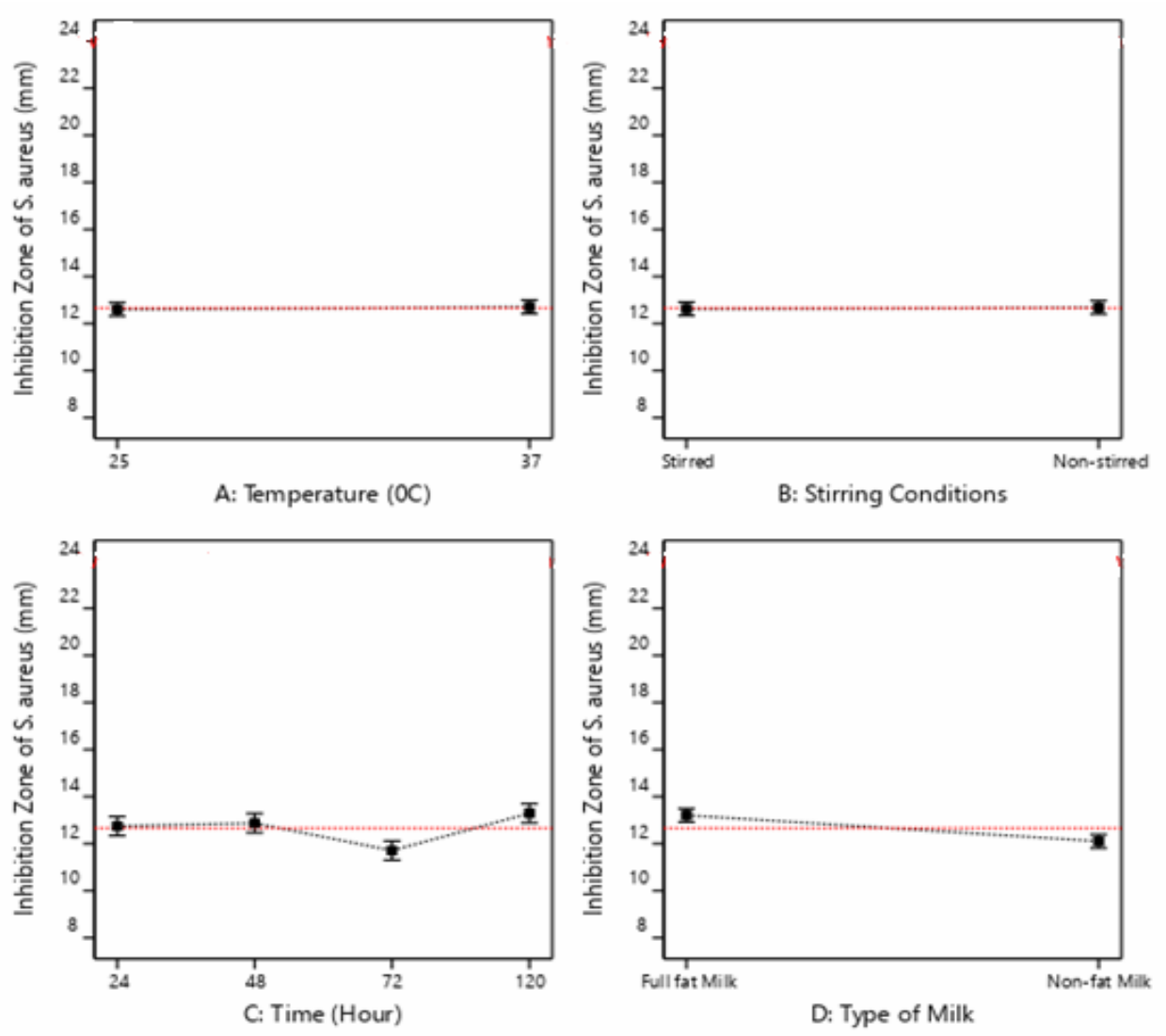

Fig. 1. Effects of fermentation conditions on inhibition zone of *S. aureus* in Kefiran samples (Factors involved in multiple interactions)

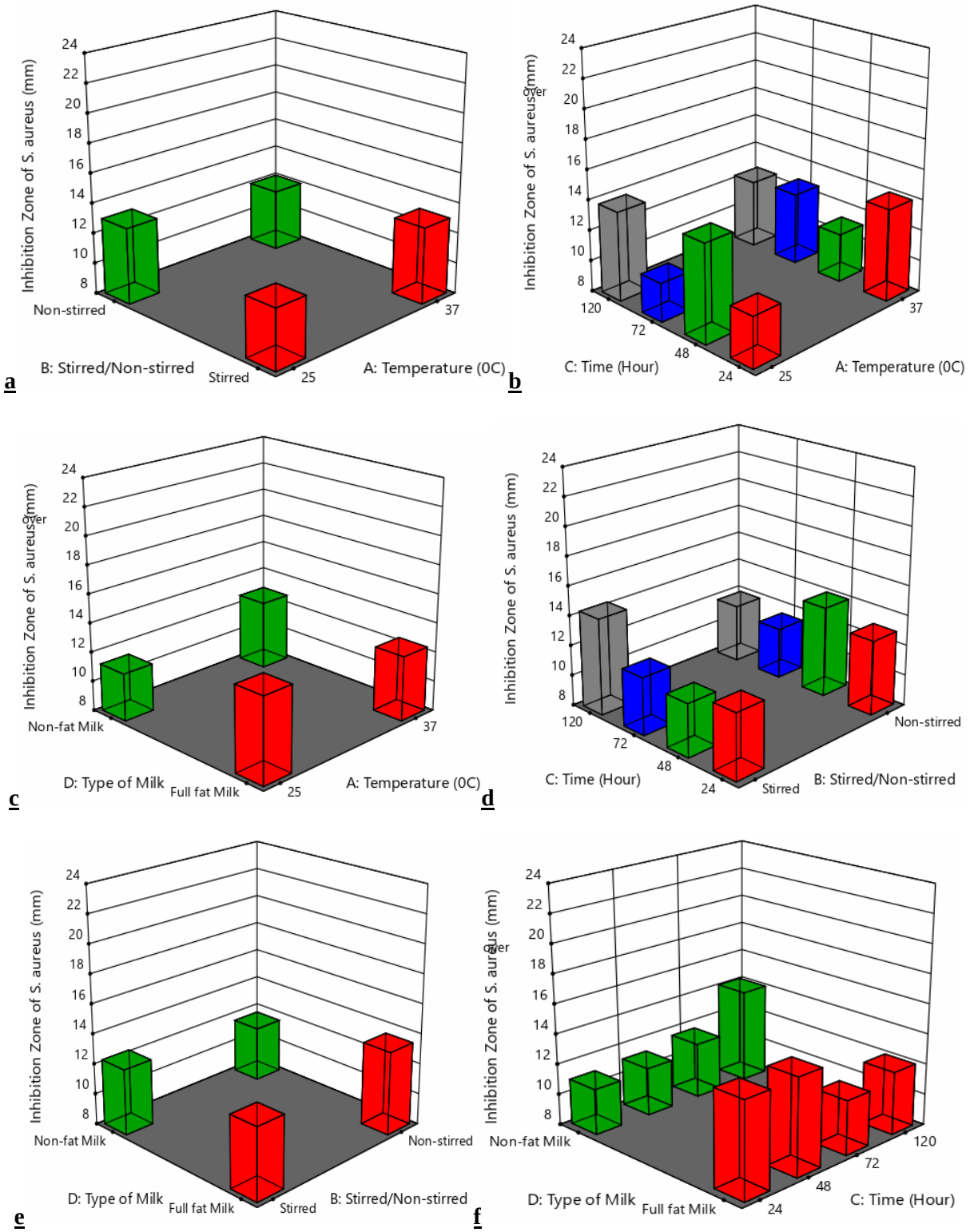

Fig. 2. Interaction effects of fermentation conditions on inhibition zone of *S. aureus* in Kefiran samples (Stirred and Non-stirred in 25 and 37°C (**a**), Different times in 25 and 37°C (**b**), Full fat and Non-fat milk in 25 and 37°C (**c**) Stirred and Non-stirred in different times (**d**), Full fat and Non-fat milk stirred and Non-stirred (**e**) and Full fat and Non-fat milk in different times (**f**)

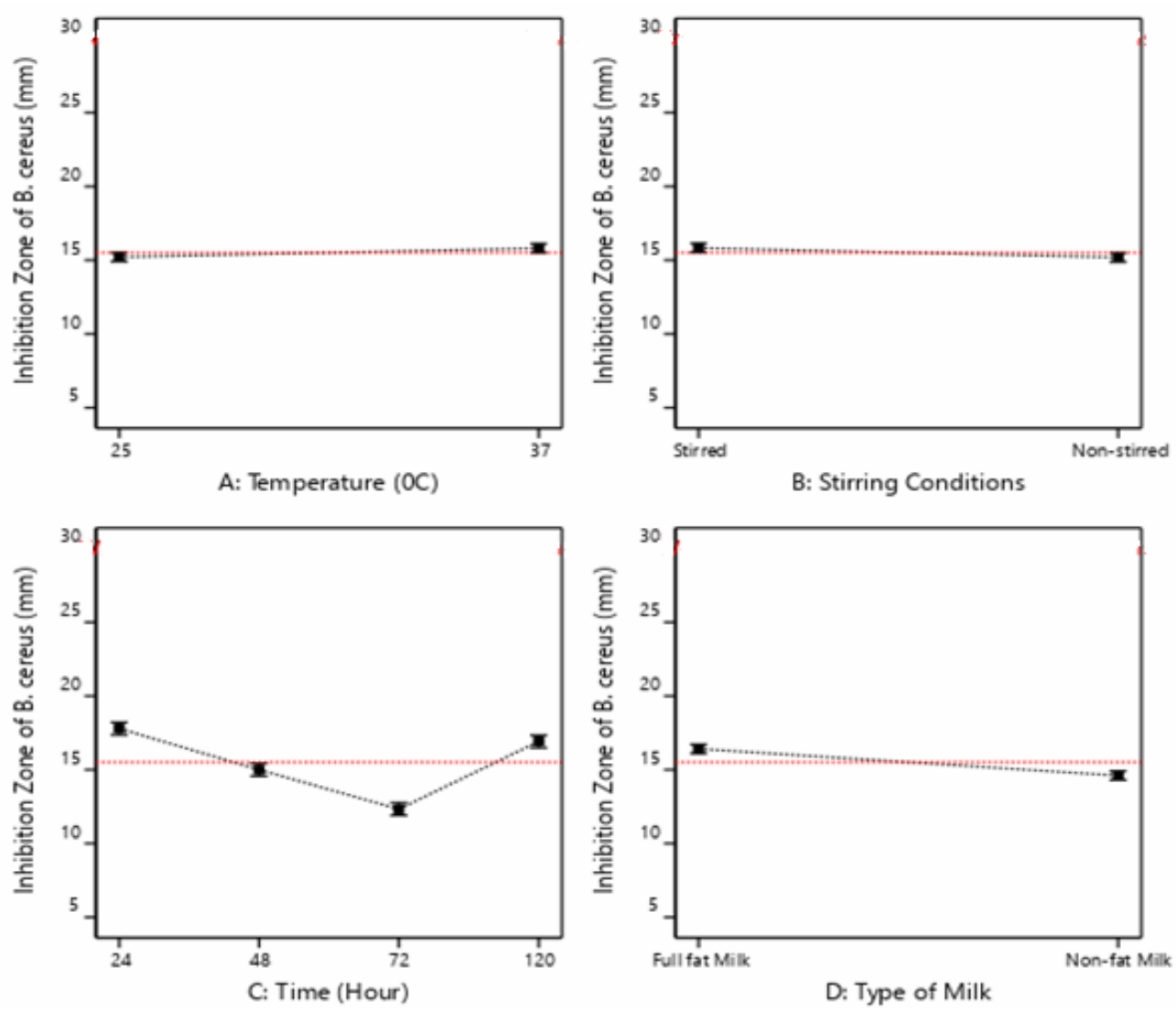

Fig. 3. Effects of fermentation conditions on inhibition zone of *B. cereus* in Kefiran samples (Factors involved in multiple interactions)

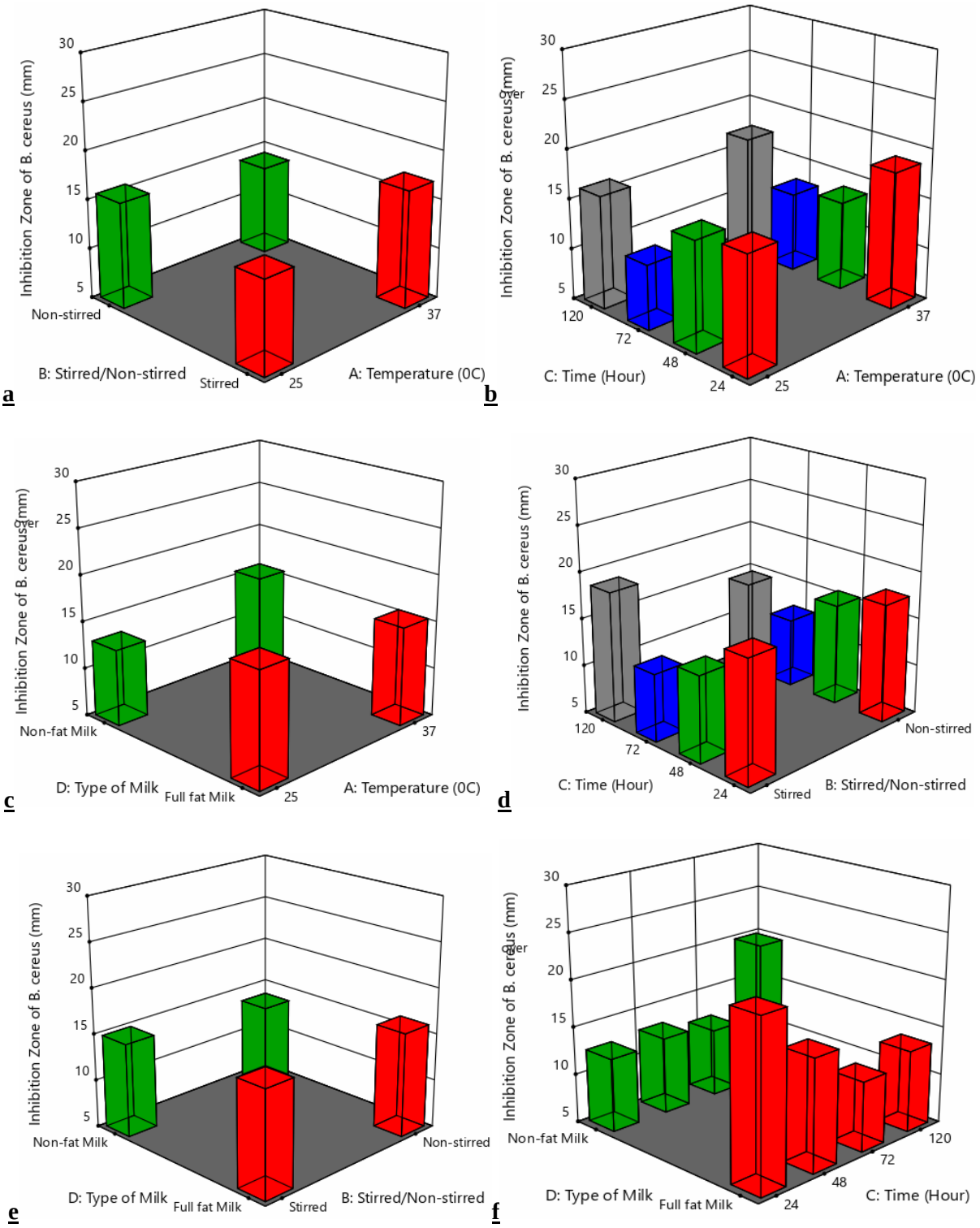

Fig. 4. Interaction effects of fermentation conditions on inhibition zone of *B. cereus* in Kefiran samples (Stirred and Non-stirred in 25 and 37°C (**a**), Different times in 25 and 37°C (**b**), Full fat and Non-fat milk in 25 and 37°C (**c**) Stirred and Non-stirred in different times (**d**), Full fat and Non-fat milk stirred and Non-stirred (**e**) and Full fat and Non-fat milk in different times (**f**)

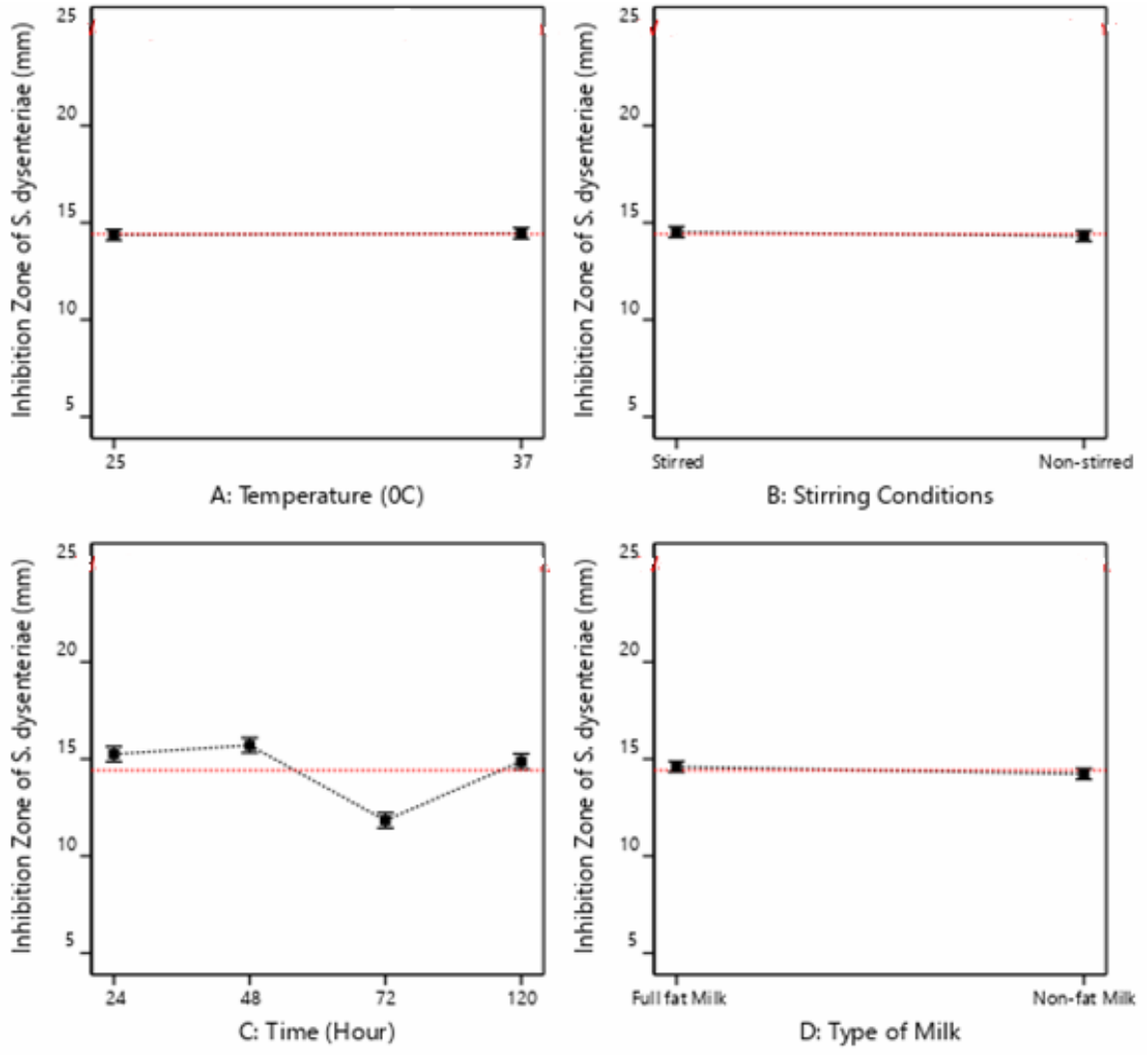

Fig. 5. Effects of fermentation conditions on inhibition zone of *S. dysenteriae* in Kefiran samples (Factors involved in multiple interactions)

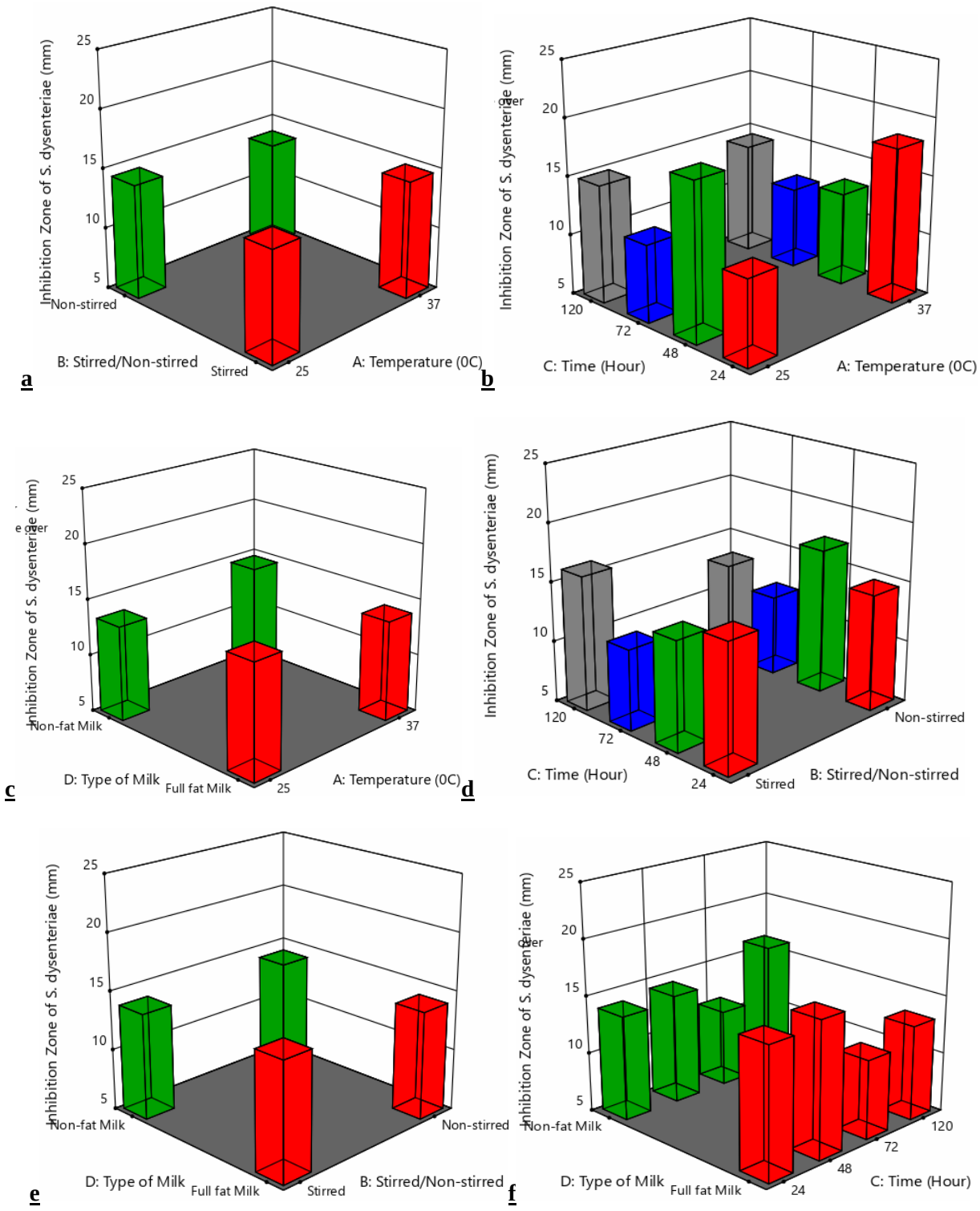

Fig. 6. Interaction effects of fermentation conditions on inhibition zone of *S. dysenteriae* in Kefiran samples (Stirred and Non-stirred in 25 and 37°C (**a**), Different times in 25 and 37°C (**b**), Full fat and Non-fat milk in 25 and 37°C (**c**) Stirred and Non-stirred in different times (**d**), Full fat and Non-fat milk stirred and Non-stirred (**e**) and Full fat and Non-fat milk in different times (**f**)

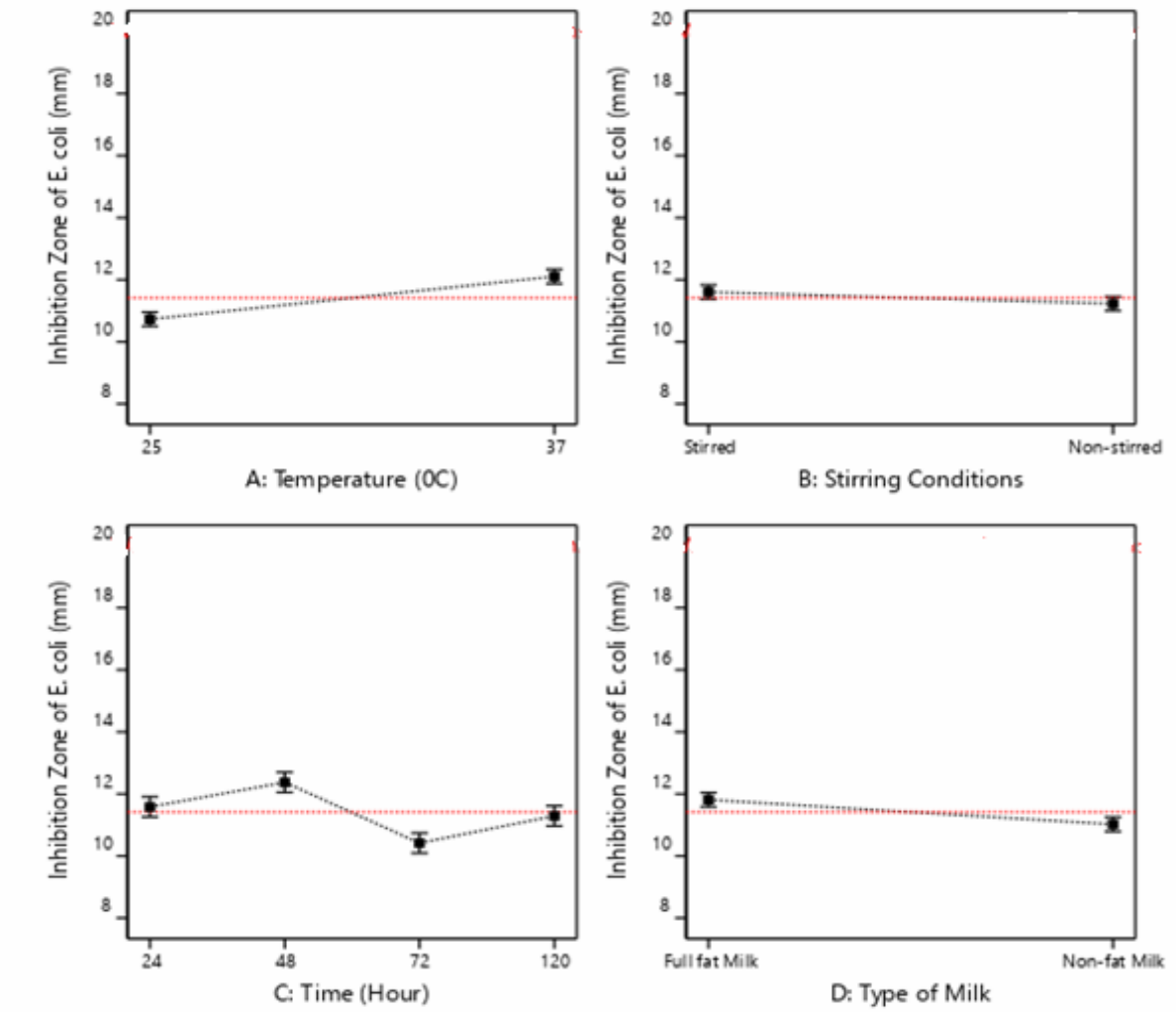

Fig. 7. Effects of fermentation conditions on inhibition zone of *E. coli* in Kefiran samples (Factors involved in multiple interactions)

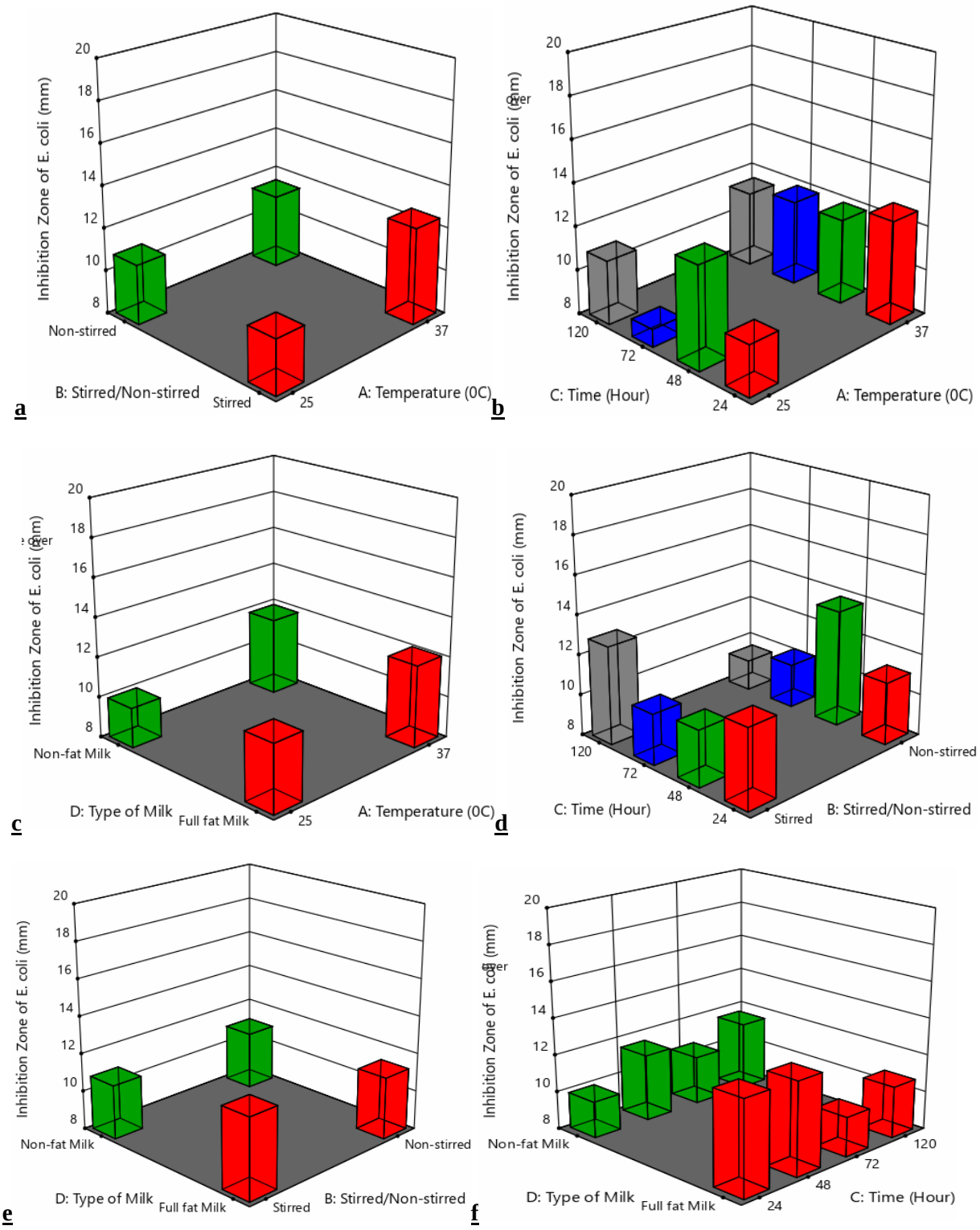

Fig. 8. Interaction effects of fermentation conditions on inhibition zone of *E. coli* in Kefiran samples (Stirred and Non-stirred in 25 and 37°C (**a**), Different times in 25 and 37°C (**b**), Full fat and Non-fat milk in 25 and 37°C (**c**) Stirred and Non-stirred in different times (**d**), Full fat and Non-fat milk stirred and Non-stirred (**e**) and Full fat and Non-fat milk in different times (**f**)

3. 6. MIC and MBC of extracted kefiran

Other objectives of the present study were to determine the MIC and MBC of extracted kefiran. Samples of extracted kefiran that showed the highest the inhibition zone diameter in well method were selected for MIC and MBC analysis. For this purpose, were used extracted kefiran samples from fermented kefir grains in full-fat milk, under stirred conditions at 25°C for 48 hours.

MIC and MBC of extracted kefiran samples were determined for the tested bacteria in the range of 1.4-11.25 mg/ml (Table 4).

Table 4. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of extracted kefiran (mg/ml)

Bacteria	MIC	MBC
S. dysenteriae	1.4	1.4
S. aureus	5.6	5.6
E. coli	11.25	11.25
B. cereus	2.8	2.8

Rodrigues et al reported MIC and MBC levels of extracted kefiran from kefir grains of 4.62 and 4.94 mg/ml, respectively. The bacterium of Gram-positive of *S. pyogenes* was reported to be the most susceptible and *S. aureus*, *S. salivarius*, *S. typhimurium*, *C. albicans* and *L. monocytogenes* were in the next susceptibility class. *P. aeruginosa* and *E. coli* were identified as the most resistant to extracted kefiran from kefir grains (Rodrigues et al., 2005). In the present study, *E. coli* with MIC and MBC of 11.25 mg/ml was identified as the most resistant to the extracted kefiran.

Of particular note in the study of Rodriguez et al. is the proximity of the MIC and MBC that were also observed in the present study (Rodrigues et al., 2005). The similarity of MIC and MBC levels or the proximity between the two indicates the lethal activity of tested compound. Rezaei et al. Reported the antibacterial activity of extracted kefiran from kefir grains against Gram-positive bacteria of *B. cereus*, *S. aureus* and *L. monocytogenes*. They were also observed resistance of *E. coli* and *P. aeruginosa* to extracted kefiran from kefir grains (Rezaei et al., 2012).

The more resistance of Gram-negative bacteria such as *E. coli* are attributed to their cell wall structure and its lower permeability to various chemical compounds with an antimicrobial nature. The existence of lipopolysaccharide layer in the cell wall and extensive periplasmic space in Gram-negative bacteria are one of the important reasons for *E. coli* resistance (Nikaido., 2003).

S. dysenteriae is also a Gram-negative bacterium. The significant sensitivity of this Gram-negative bacterium to the extracted kefiran samples was interesting. This sensitivity in similar studies relates to the specific differences of each microorganism species (Hizomi et al., 2018; Ceyhan and Ugur., 2001; Tumin et al., 2005; Taormina et al., 2001).

4. Conclusion

In general, the kefiran extracted from fermented kefir grains in full-fat milk showed higher antibacterial activity. Also, to achieve the highest antibacterial activity against *B. cereus, E. coli*, *S. dysenteriae,* and *S. aureus* is recommended fermentation time for 24, 48, 48 and 120 hours, respectively. Fermentation temperature and stirring conditions had no significant effect on the antibacterial activity of the extracted kefiran samples.

Conflict of interest

The authors declare that there is no conflict of interest.

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