Research Article

Chemical Review and Letters
journal homepage: www.chemrevlett.com
ISSN (online): 2645-4947 (print) 2676-7279

Co-production of conjugated linoleic acids, exopolysaccharides and bacteriocins by *Lactobacillus acidophilus* LA5 and *Bifidobacterium animalis* subsp. lactis BB12 in supplemented dairy effluents

Saber Amiri [a*], Mahmoud Rezazadeh Bari [a], Mohammad Alizadeh Khaledabad [a], Reza Rezaei Mokarram [b], Mahmoud Sowti Khiabani [b]

[a] *Department of Food Science and Technology, Faculty of Agriculture and Natural Resources, Urmia University, P.O. Box 57561-51818, Urmia, Iran*
[b] *Department of Food Science and Technology, Faculty of Agriculture, University of Tabriz, Tabriz, Iran*

ARTICLE INFO

Article history:
Received 21 November 2020
Received in revised form 1 January 2021
Accepted 10 January 2021
Available online 25 January 2021

Keywords:
Postbiotics
Conjugated linoleic acids
Exopolysaccharides
Bacteriocins
Probiotics
Dairy effluents

ABSTRACT

In this study, the effects of initial pH (5 − 7), temperature (30 − 38 ◦C) and incubation time (12 − 48 h), as well as yeast extract and free linoleic acid concentrations, respectively (0 – 4 %) and (0 − 400 µL), on the co-production of conjugated linoleic acid (CLA), exopolysaccharides (EPSs) and bacteriocins (BACs) by *Lactobacillus acidophilus* LA5 and *Bifidobacterium animalis* subsp. lactis BB12 and their biomass in cheese whey and milk permeate were evaluated. The results showed that biomass, CLA, EPSs and BACs activity ranged Log 0.80 - Log 8.67 $g.L^{-1}$, 3.08-107.95 $\mu g.mL^{-1}$, 107.75-351.92 $mg.L^{-1}$ and 9.29-14.62 mm, respectively. Yeast extract concentration was the only factor with the positive significant effect on biomass and postbiotic metabolites i.e. its increasing caused to an increase in both of them ($p<0.05$). The temperature significantly affected the production of biomass and CLA; its increasing resulted in increasing both ($p<0.05$). The initial pH had significant, but different, effects on EPSs and BACs production ($p<0.05$) i.e. EPSs and BACs production decreased and increased, respectively, as a result of increased initial pH. Increasing free linoleic acid concentration from 0 up to 400 µL led to increased CLA biosynthesis. Higher biomass, EPSs and BACs are produced in cheese whey, compared with milk permeate, but CLA produced in milk permeate was higher than that obtained in cheese whey. *B. animalis* BB12 produced more biomass, CLA and EPSs in comparison to *L. acidophilus* LA5. However, these probiotics had no statistical difference in terms of producing BACs. This work successfully demonstrated the co-production potential of CLA, EPSs and BACs by two commercial probiotics in dairy effluents.

1. Introduction

In the recent decades, due to consumers' interest in the functional and safe food, the incorporation of probiotic cells in different foodstuffs is progressively increasing [1-8]. In the food industry, especially in dairy products the most commonly-used genera of probiotic bacteria are *Lactobacilli* and *Bifidobacteria*, the main populations of microbiota in the humans' small and large intestine, respectively. In addition, generally used probiotic bacteria in the food industry are in the form of direct vat set, freeze dried culture concentrates. According to the most accepted definitions, probiotics are live microorganisms with health benefits to host when consumed in adequate amounts and considered as GRAS (Generally Recognized as Safe) [9-13].

Enhancing the functionality of intestinal barrier and regulating the immune system as well as improving certain gastrointestinal disorders, such as irritable bowel syndrome, inflammatory bowel disease, and the defense system by competing against pathogens are examples of health effects of probiotics on the host [13].

* Corresponding author: Saber Amiri; e-mail address: St_sa.amiri@urmia.ac.ir

The term "Postbiotics" is referred to any bioactive molecules with health-beneficial effects, produced by probiotic bacteria. These metabolites include bioactive lipids such as conjugated linoleic acid, antimicrobials like bacteriocins and exopolysaccharides [14].

Conjugated linoleic acids (CLA) are defined as a set of linoleic acid (LA; C18:2) isomers with conjugated bonds, biosynthesized through the bio-hydrogenation process by microorganisms during the exponential phase. Due to their bioactive properties, *cis 9-trans 11, trans 10-cis 12* and *trans 9- trans 11* are very important among the CLA isomers. According to some recent reports, these isomers have a plenty of health benefits such as anti-inflammatory, anti-diabetic, anti-cancer, immuno-modulatory, anti-atherosclerotic, and anti-obesity activities [13, 15, 16].

As the primary metabolites, bacterial Exopolysaccharides (EPSs) are characterized as polysaccharides molecules, secreted by some bacteria into the medium [17, 18]. Although most studies focused on the EPSs industrial applications due to their textural properties, bioactive properties of EPSs have attracted much attention in recent years. Also, the most recent studies demonstrated that EPSs have immunomodulatory potential, and anti-inflammatory, anti-biofilm and antioxidant activities [17-19].

Bacteriocins (BACs) are ribosomally bio-synthesized small bacterial peptides. These primary metabolites have antagonistic activity against bacteria genetically similar to the producing bacteria. As natural food bio-preservatives, used in food products to biological control of spoilage and pathogenic bacteria, bacteriocins get increasing interests. In addition, currently, bacteriocins have been considered as bioactive molecules with potential activities such as antiviral agents and anticancer agents [20-22].

In the last decades, utilization of different agricultural residual and industrial wastes as substitute for nutritious sources of microbial fermentation have attracted much attention of researchers [23]. Cheese whey and milk permeate are main effluents of cheese producing industry and ultrafiltration of milk for ultra-filtered white cheese production, respectively, in Iran. These low-cost by-products are available in large quantities and can be used as substrates due to their high concentrations of lactose and other nutrients [20, 24].

The ability of various probiotics to individually production of CLA [13, 15, 16, 25], EPSs [17-19, 26] and BACs [20, 22, 24, 27] have been investigated; however, there is no report about co-production of these postbiotic metabolites. Therefore, the main objectives of this study were to compare *Bifidobacterium animalis* subsp. lactis BB12 and *Lactobacillus acidophilus* LA5 single culture for production of CLA, EPSs and BACs in cheese whey and milk permeate as the media, and investigate the effects of incubation time, incubation temperature, initial medium pH, and study the impacts of adding yeast extract and free linoleic acid as supplements to cheese whey and milk permeate on the co-production of these postbiotic metabolites.

2. Materials and methods

2.1. Bacterial strains and inoculation cultures

Freeze dried cultures *L. acidophilus* LA5 and *B. animalis* subsp. *lactis* BB12 (Chr. Hansen, DK-2970 Hørsholm, Denmark) were obtained and individually weighted, as recommended by the manufacturer, then grown for 24 h at 37 ◦C in de Man, Rogosa, and Sharpe (MRS) broth (Merck, Darmstadt, Germany) + tween 80 (0.1%, AppliChem, Darmstadt, Germany) and in MRS + tween 80 (0.1%) + L-cysteine (0.05%, AppliChem, Darmstadt, Germany) + lithium chloride (0.1%, Sigma-Aldrich, St. Louise, Missouri, USA), respectively. Then, the cell cultures (30 mL) were centrifuged (2360 × g) for 8 min and washed twice in NaCl solution (0.85%). The pellet was suspended again in the saline solution to obtain a suspension containing 10^9 CFU.mL^{-1} [28].

2.2. Media preparation and growth conditions

Both cheese whey (CW) and milk permeate (MP), obtained from local dairy plants, were prepared as follows: after adjusting the pH to 4.5 with 5 mol.L^{-1} HCl, they were heated (121 ◦C for 15 min) to denature the proteins. Then, the precipitates were removed by filtration (Whatman No. 5, Whatman International Ltd., Maidstone, UK). After that, following the adjustment of the pH of supernatants, according to experiment, they were sterilized at 121 ◦C for 15 min. Subsequently, cellulose acetate membrane filters with 0.45 µm pore size used to add linoleic acid (99% Sigma-Aldrich, St. Louise, Missouri, USA) in 2% Tween 80 and yeast extract (Sigma-Aldrich, St. Louise, Missouri, USA) depending on the experiment. Batch fermentations of pure probiotic culture were performed in 100 mL Erlenmeyer flasks with 50 mL media by 10^9 CFU.mL^{-1} inoculum and incubated in at different incubation temperatures and times according to the experiment [29].

2.3. Biomass determination

To measure biomass, the cells were harvested by centrifugation at 5000 × g for 15 min at 4 ◦C from culture media and washed twice with sterile normal saline (0.85% NaCl). Then, the calibration curve of OD_{600} vs dry weight was drawn and used in measurement of biomass [30].

2.4. Extraction and quantitative determination of CLA

In order to extract CLA from culture media, 10 mL of culture media were centrifuged at 6300 × g for 5 min at 4 ◦C. Then, 3 mL of the supernatant were added to 6 mL

of isopropanol and vortexed for 1 min. After that, 5 mL of n-hexane was added and vortexed for 1 min and centrifuged at 448 × g for 5 min at 4 ◦C.

Finally, total CLA measurements were carried out in triplicate for 2 mL of the CLA extract in quartz cuvettes by n-hexane as a blank at 233 nm, using an Ultraviolet–visible spectrophotometer (80-2088-64, Pharmacia LKB Biochrom, Cambridge, UK). The standard curve was constructed for 0 – 30 ($mg.mL^{-1}$ in 2% Tween 80) concentration of CLA (99%, Sigma-Aldrich, St. Louise, Missouri, USA) at 233 nm [31].

2.5. Isolation and quantitative determination of EPSs

To isolate EPSs from culture media, 5 mL of culture media were centrifuged at 2800 × g for 30 min at 4 ◦C. Then, 5 mL of tricarboxylic acid was added to the supernatant in order to inactivate EPSs-degrading enzymes, while cold ethanol was added for the precipitate proteins and concentrate the polysaccharides. After that, EPSs were dissolved in deionized water and then dialyzed in distilled water. Measurements of total EPSs were carried out in triplicate by the phenol-sulfuric acid method, widely used for this purpose. The standard curve was constructed for 0 − 150 $mg.L^{-1}$ concentrations of glucose (Merck, Darmstadt, Germany) at 500 nm [32].

2.6. Purification and determination of bacteriocins' activity

In order to purify the bacteriocins, one mL of culture medium was centrifuged at 10000 × g for 10 min at 4 ◦C. The supernatant was filtered through syringe filters with 0.45 µm pore size (Supor® membrane, Paul Co. Ltd., Ann Arbor, MI). Then, the activity of bacteriocins was estimated using the agar well-diffusion method as described below. First, Brain heart infusion agar (Merck, Darmstadt, Germany) was cooled to 47 ◦C and inoculated with 1 ml overnight culture containing 10^8 $CFU.mL^{-1}$ of *Listeria monocytogenese* ATCC 19113 as an indicator strain. Then, it was poured into a sterile plate at room temperature. After solidification, wells with 6 mm in diameter were cut and filled with 50 µl of supernatant neutralized to pH 7 with 1 $mol.L^{-1}$ NaOH solution. The plates were kept in the refrigerator (4 ◦C) for 2 h to diffuse supernatant and then incubated at 37 ◦C for 24 h. Finally, the inhibition zone diameters were determined [20].

2.7. Experimental design and statistical analysis

A factorial experimental design was applied to investigate the effects of five numerical variables including initial pH, temperature, incubation time, linoleic acid and yeast extract concentrations, as well as two categorical variables, consisting of types of culture media and bacterial cultures, on co-production of postbiotic metabolites (Supplemental file 1, Table 1 & Table 2). ANOVA statistical analysis was utilized to evaluate the statistical significance of the different independent variables and their interactions by Fisher's F-test and p-value at $\alpha \geq 0.05$. The design and statistical analysis, as well as charting, were done by Design-Expert Version 10.0.4.0 (Stat-Ease, Int. Co., Minneapolis, MN, USA).

3. Results

Generally, production of CLA, EPSs and BACs are dependent on different environmental factors and producer bacteria [14]. Therefore, the effects of different parameters including initial pH, incubation temperature, incubation time and type of commercial probiotic bacteria as well as nutritional supplementation on co-production of these postbiotic metabolites and probiotics biomass were investigated.

3.1. Growth of probiotic bacteria

Figure 1a - d shows the effects of investigated parameters on the biomass of *L. acidophilus* LA5 and *B. animalis* subsp. *lactis* BB12 during the fermentation. According to the results, temperature, yeast extract concentrations, type of culture medium and type of bacterial cultures had significant effect on biomass ($p<0.05$). However, the biomass was not significantly affected by the initial pH, incubation time and linoleic acid concentrations ($p>0.05$). Increasing temperature from 30°C to 38°C caused to the increased biomass of BB12 and LA5. Biomass of both probiotic bacteria increased by an increase in the yeast extract concentrations (Supplemental file 2a).

Although biomass of BB12 in CW was higher than that of it in MP, biomass of LA5 had no difference in CW and MP (Fig. 1a). Furthermore, Fig. 1a, illustrates that the biomass of BB12 was significantly higher than that of LA5. In pH higher than 5, biomass increased by rising temperature.

Nevertheless, in pH 5, increasing temperature had no effect on biomass (Supplemental file 2b). Although biomass was constant in CW by increasing pH from 5 to 7, it increased in MP (Fig. 1b). Additionally, Fig. 1b, depicts that in the pHs between 5 to 5.75, biomass in CW was higher than that in MP. However, in the pH range of 5.75 to 7, biomass in MP was higher than that in CW. Figure 1c, shows the interaction of initial pH and type of probiotic bacteria, according to which, biomass of BB12 was higher than LA5 in constant pH. Accordingly, by increasing the pH, while the biomass of BB12 decreased, that of LA5 remained constant. As observed in, the interaction of incubation time with type of probiotic bacteria is shown in Fig. 1d, clearly, the biomass of BB12 was higher than LA5 in constant fermentation time. In addition, by increasing fermentation time from 12 h to 48 h, the biomass of BB12 and LA5 was decreased and increased, respectively.

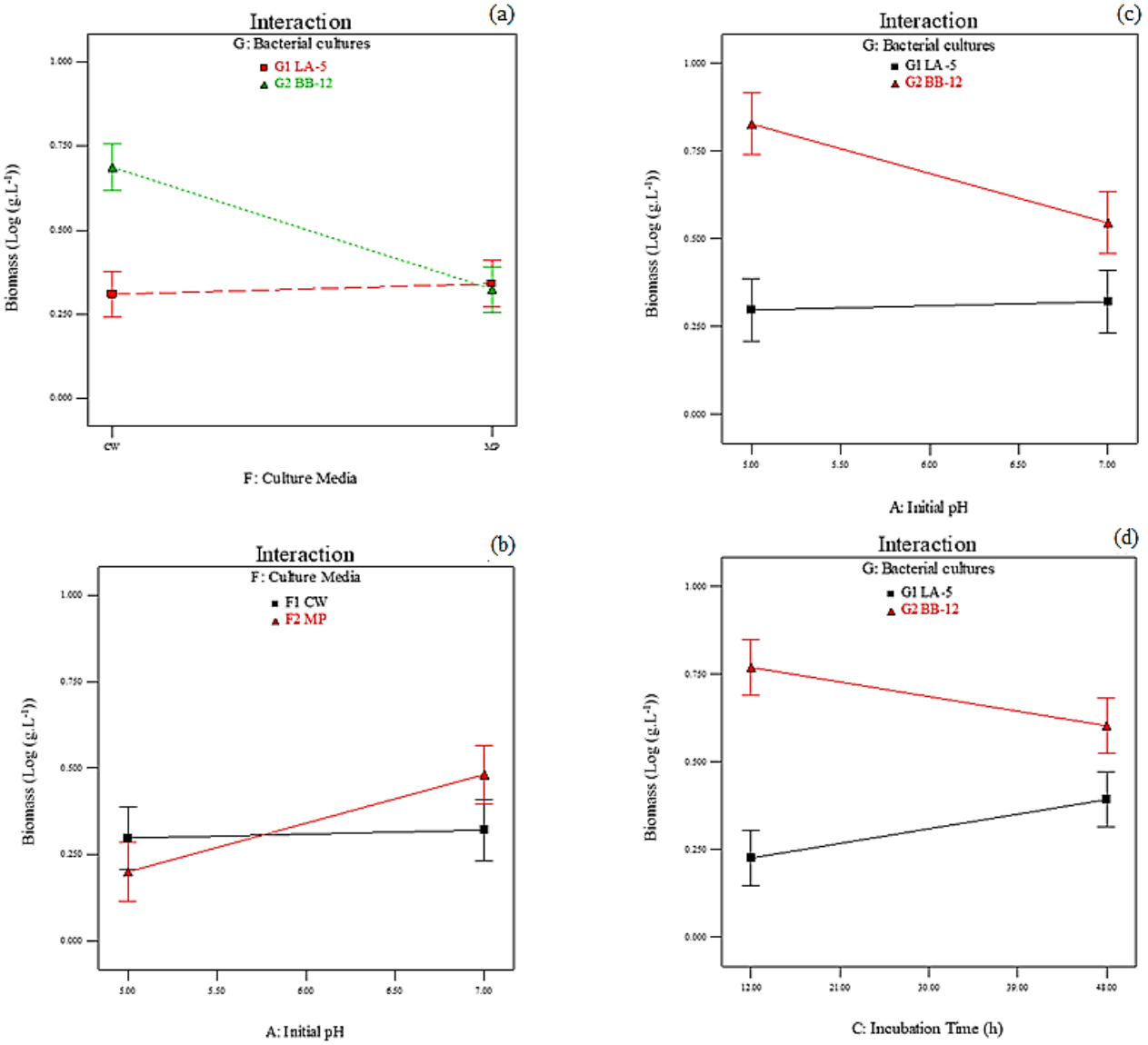


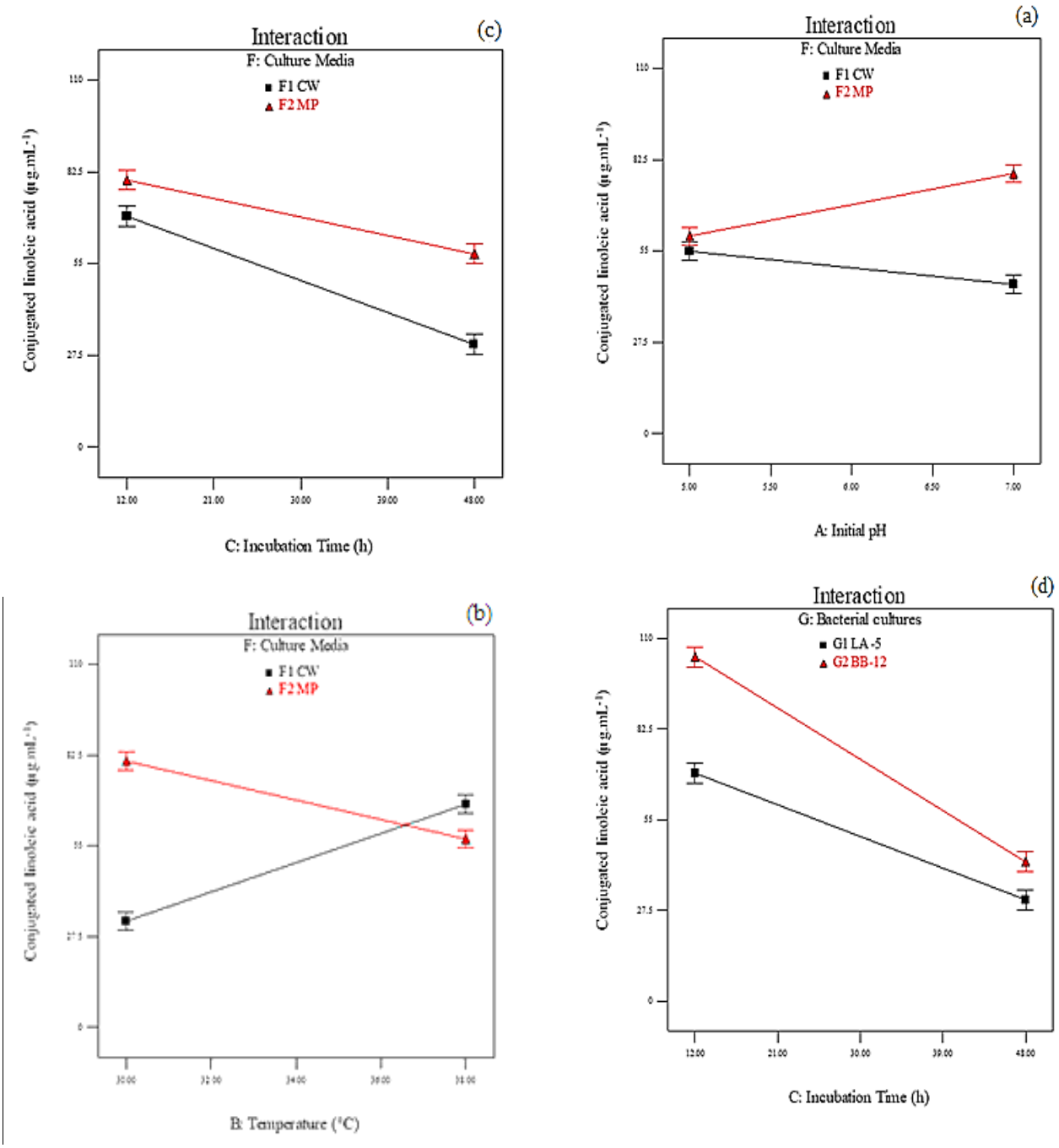
Fig. 1: The effects of independent variables on biomass of probiotic bacteria

3.2. CLA production

According to the obtained results, the effects of temperature, incubation time, linoleic acid and yeast extract concentrations as well as types of culture media and probiotic bacteria on the CLA production were statistically significant ($p<0.05$). The values of CLA produced during the fermentation process are shown in Fig. 2a - f. According to results, the CLA production witnessed an increased by increasing the free linoleic acid concentration from 0 $\mu g.mL^{-1}$ to 400 $\mu g.mL^{-1}$. Furthermore, increasing the concentration of yeast extract from 0% to 4% caused to higher CLA production. Although CLA production increased by rising temperature from 30°C to 38°C, its production decreased by increasing the incubation time from 12 h to 48h (Supplemental file 3a). In constant pH, CLA production increased by increasing temperature. However, it decreased by increasing the incubation time (Supplemental file 3b). At 38 ◦C, by increasing pH from 5 to 7, CLA biosynthesize decreased, but at 30 ◦C, it had no significant effect on CLA value. Increasing the initial pH caused to an increase in the CLA production by adding yeast extract, whereas without yeast extract, increasing the initial pH resulted in lower CLA production (Supplemental file 3c). Figure 2a, compares the CLA production in CW and MP, confirming that in pH 5, CLA value in two media had no difference. However, by increasing pH from 5 to 7, its value increased and decreased in MP and CW, respectively. At constant temperature, CLA biosynthesize value after 12h fermentation was higher than that of it after 48h fermentation (Supplemental file 3d). CLA production increased by increasing free linoleic acid concentration and temperature (Supplemental file 3a & 3d). Besides, increasing yeast extract concentration and temperature caused to an increase in the CLA bio-production. However, at 38 ◦C, the bioconversion free linoleic acid to CLA was not affected by yeast extract concentration (Supplemental file 3e). As can be seen from the Fig. 2b, the effect of culture media type on the CLA production

was dependent on temperature. In addition, parallel with increasing incubation temperature, the production of CLA in CW and MP increased and decreased, respectively. Although CLA biosynthesize in MP was higher than CW at 30 – 37 ◦C, its biosynthesize in CW was higher than MP at 37 – 38 ◦C. Increasing the incubation time led to a reduced CLA content in the investigated concentrations of yeast extract (0 − 4 %). Furthermore, after 48 h of incubation, the produced CLA showed no difference in 0 − 4 % of yeast extract. However, at lower incubation time, the maximum CLA production is observed in 4% yeast extract (Supplemental file 3f). As can be observed in Fig. 2c, CLA production in MP is higher than CW at constant incubation time. Nonetheless, CLA content was decreased in both media by increasing incubation time. Figure 2d, shows that at constant incubation time, the CLA production ability of BB12 is higher than LA5. However, CLA production ability of both probiotics is decreased by increasing incubation time. The Fig. 2e, indicates that CLA biosynthesize in both CW and MP was increased by increasing free linoleic acid, even though at 400 µg.mL^{-1} of free linoleic acid, bio-conversed CLA content was equal in both media and had no significant difference.

From Fig. 2f, CLA production ability of BB12 and LA5 can be compared with each other; BB12 produced higher CLA value compared with LA5 at constant concentration of yeast extract. It is apparent from this figure, that CLA produced in CW by BB12 was higher than that produced by LA5. Nonetheless, both BB12 and LA5 had same quantity of produced CLA in MP.



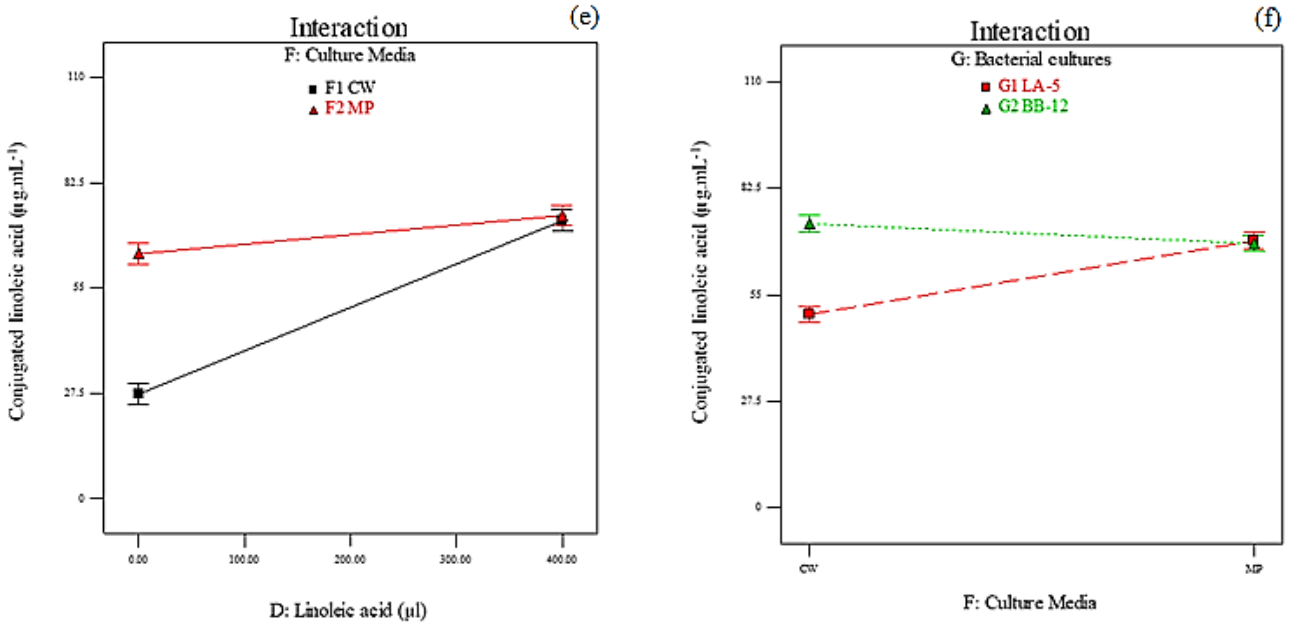


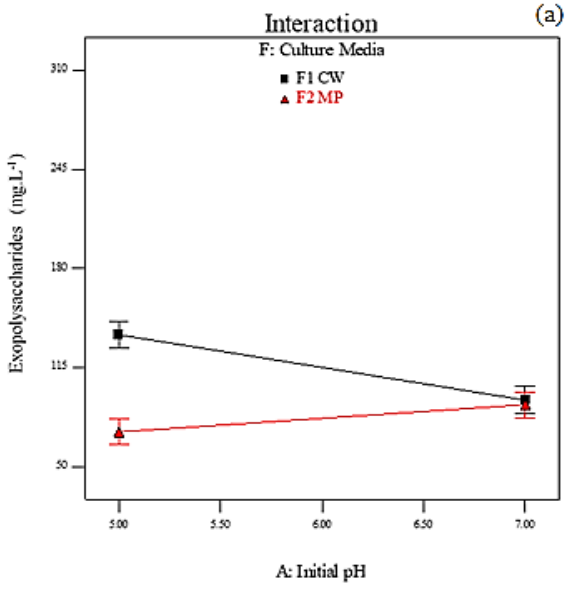
Fig. 2: The effects of independent variables on CLA bio-synthesis

3.3. EPSs production

According the results, initial pH, yeast extract concentration, type of culture media and probiotic bacteria significantly affected EPSs production ($p<0.05$). Results showed that EPSs production was significantly decreased and increased, respectively, by increasing initial pH and yeast extract concentration. In addition, the amount of EPSs in CW was higher than EPSs produced in MP. Furthermore, the ability of BB12 to produce EPSs was higher that of LA5.

Figure 3a, b, c, d and e reveals that EPSs production was affected by significant factors. Increasing pH without adding yeast extract had no effect on EPSs production, but it caused to a decrease in production of EPSs by adding 4% yeast extract (Supplemental file 4a).

As confirmed by the Fig. 3a, an increase in pH resulted in a decrease and increase in the production of EPSs in CW and MP, respectively. From this figure, although the amount of EPSs in two media was equal in pH 7, its amount was higher in CW in lower pH. Figure 3b illustrates the interaction of initial pH with probiotic bacteria, showing that increasing pH has negative effect on EPSs production ability of both bacteria. Besides, EPSs production by BB12 and LA5 decreased by increasing pH in the investigated range. Increasing temperature and adding 4% yeast extract clearly ended in an increase in the production of EPSs. However, without adding yeast extract, the production of EPSs is not affect by increasing temperature (Supplemental file 4b). The Fig. 3c illustrates that the amount of EPSs is not affected by type of culture media in 30 ◦C, but the amount of EPSs in CW and MP increased and decreased by increasing temperature, respectively. The production of EPSs by both probiotics (BB12 and LA5) increased by increasing temperature of fermentation process (Fig. 3d). By increasing fermentation time, without adding yeast extract, the EPSs production decreased, but by increasing concentration of yeast extract up to 4%, the EPSs production increased (Supplemental file 4c). The effect of yeast extract on EPSs production can be observed in Fig. 3e, showing that EPSs produced in two media had no statistically difference without yeast extract supplementation. Nonetheless, the higher production of EPSs was witnessed by adding yeast extract to both media. Moreover, the increase observed in CW was higher than MP. Figure 3 compares the EPSs producing ability of investigated probiotics, demonstrating that in both media, BB12 could produce higher levels of EPSs in comparison with LA5. In this figure can be seen that both of probiotic bacteria could produce higher amounts of EPSs in CW, as culture media, compared with MP.



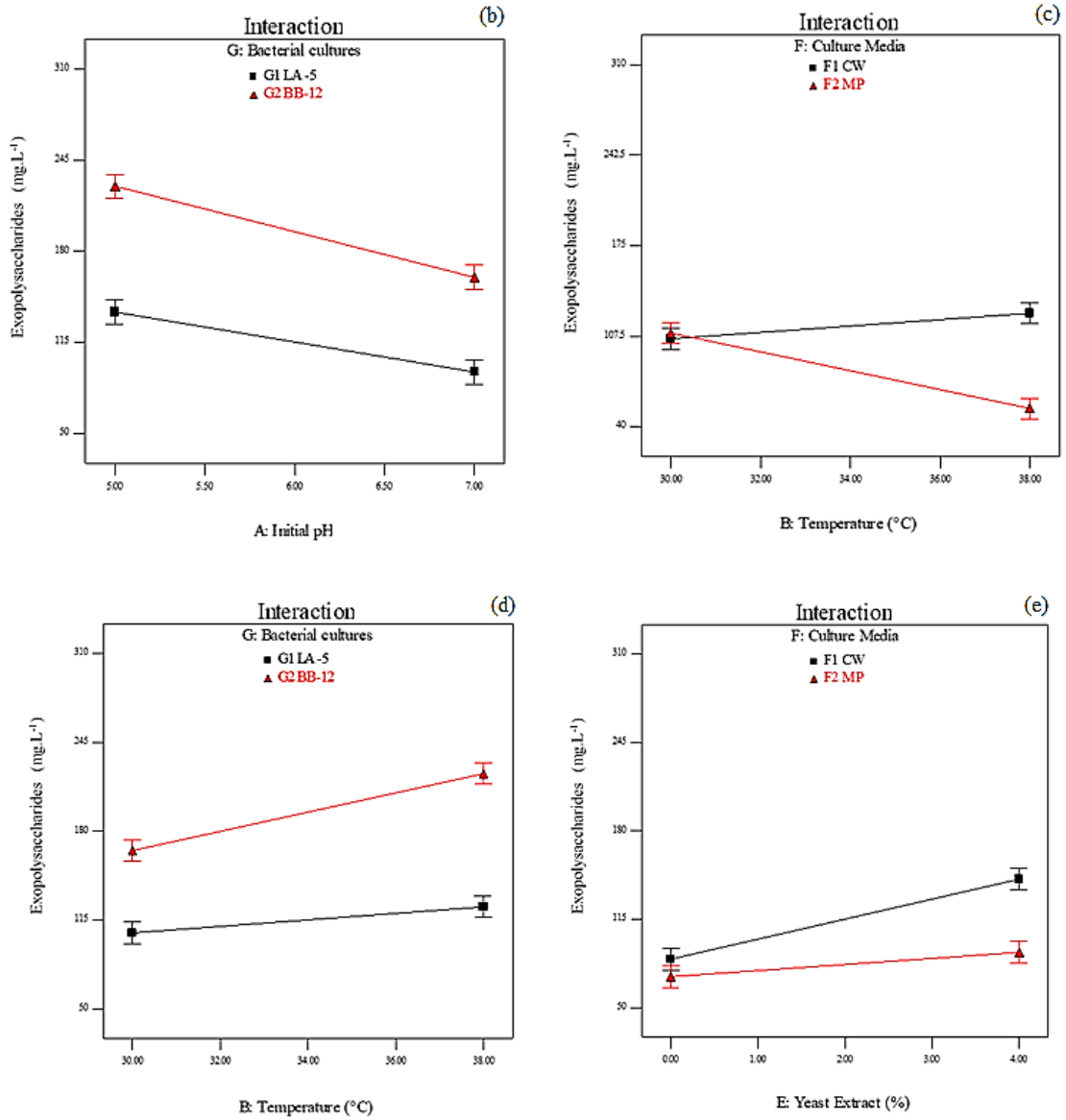


Fig. 3: The effects of independent variables on EPSs bio-production

3.4. BACs activity

The results showed that the initial pH, temperature, incubation time, yeast extract concentration and type of culture media had significant effect on bacteriocins production. The initial pH, temperature and yeast extract concentration had positive effect i.e. increasing these parameters led to increased bacteriocins activity. On the other hand, incubation time had negative effect i.e. the bacteriocins activity decreased by increasing the fermentation bioprocess time. Furthermore, bacteriocins activity in CW was higher than that in MP. At constant temperature, the inhibition zone at 12 h after bioprocess was higher than that at the end of fermentation (48 h) (Supplemental file 5a). Besides, inhibition zone in diameter increased by increasing temperature, with or without supplementing yeast extract. However, by adding yeast extract, as nitrogen source, the inhibition zone increased, more than that without supplementation by yeast extract (Supplemental file 5b). As seen in Fig. 4, inhibition zone increased and decreased in CW and MP, respectively, by increasing the incubation temperature.

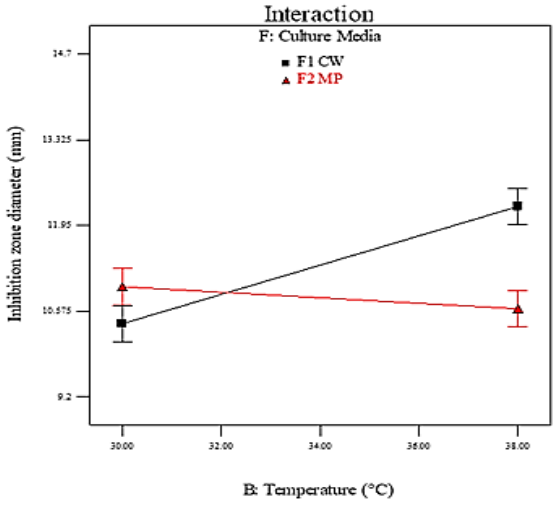


Fig. 4: The effects of independent variables on BACs' activity

4. Discussion

Although previous researches investigated and established the ability of different probiotic bacteria to production of CLA, EPSs and BAC, there is no report about co-production capacity of these bioactive metabolites by probiotics. To our knowledge, this is the first study to demonstrate the co-production potential of postbiotic metabolites by *L. acidophilus* LA5 and *B. animalis* subsp. lactis BB12 using fermentation bioprocess in CW and MP as media and investigated the effect of environmental factors.

One of the most important factors affecting both survivability and growth of probiotic strains is pH, not only because it changes surface charges of microorganisms, but also because it can affect the ionization of organic compounds in the medium and alter the pathways of microorganisms absorbing nutrients [33]. Ye et al. 2013, reported that CLA production increased from 54.17 $\mu g.mL^{-1}$ at pH 4.5 to 110.70 $\mu g.mL^{-1}$ at pH 6.5 in skim milk. Soto 2013 reported similar results, proving that CLA formation of *L. acidophilus* and ruminal bacteria increased by increasing pH from 5.5 to 6.5 [34]. Also it can be concluded that enzymatic mechanism of linoleic acid bio-conversion to CLA is highly dependent on pH of media [35]. The EPSs production is highly dependent on the pH and the pH at which EPSs formation and EPSs hydrolysis by means of glycosyl-hydrolases are balanced is the optimal pH for EPSs synthesis. Although a pH close to 6 is considered to be the optimal one for EPSs synthesis, it is specifically dependent on the species, differing in diverse species of LAB [36]. Based on the reports, EPSs are produced more efficiently at pH 5.8, whereas biomass growth is more efficient at pH 6.2, as confirmed by *L. sakei* 01. In addition, the highest EPSs is produced at pH 6.2 compared to 4.9, 5.5 and 6.9, as reported by *L. delbrueckii* subsp. *bulgaricus* strains B3 and G12 [37]. According to the findings of Gamar- Nourani et al. 1998, the optimal pH to produce the EPSs by *L. rhamnosus* C83 in a defined medium is ranging from 6.2 to7.2. Haj-Mustafa et al. (2015) investigated the effect of fermentation conditions on EPSs production by *Lactobacillus rhamnosus* 519 in skimmed milk base media and reported on the significant effect of pH and YE interaction on the production of EPSs. The findings of current study are consistent with those of Haj-Mustafa et al. 2015, who demonstrated that EPSs production increased with increasing pH, taking into consideration that the maximum EPSs was obtained around the pH 5.8 and decreased at higher pH.

Furthermore, it has been reported that EPSs production in continuously-growing cultures with controlled pH is more productive than that in acidified batch cultures. Considering the facts that pH has direct effect on both cell growth and bacteriocins production and bacteriocins production are associated with cell growth, it can be concluded that both cell growth and bacteriocins production stop at lower pH, due to the accumulation of lactic acid in medium [38].

Temperature is another important environmental factor in fermentation bioprocess [39]. The optimal temperature for the growth of *Bifidobacterium* strains is between 37 ◦C and 41 ◦C, while it is 37 ◦C for the growth of *Lactobacillus acidophilus* [21]. According to the results of Ye et al. 2013, the total amount of CLA production was significantly affected by temperature. CLA production increased from 71.30 $\mu g.mL^{-1}$ at 31 ◦C to 119.51 $\mu g.mL^{-1}$ at 37 ◦C, but its production decreased at higher temperatures. Soto 2013, showed that 37 ◦C was the best temperature for linoleic acid conversion to CLA by *L. acidophilus*. Khosravi-Darani et al. 2014, reported that CLA production increased by the addition of 4% whey powder, which could be due to the role of proteins in oxidation of linoleic acid and formation of its radical. This result is similar to previous report demonstrating that the production of CLA increased by the addition of skim milk. The role of proteins can be as hydrogen donors, increasing the isomerization of linoleic acids in bio-hydrogenation [40].

Incubation time is another environmental factor, important in fermentation bioprocess. It had significant effect on the synthesis of CLA [33]. Khosravi-Darani et al. 2014, supposed that parallel with an increase in temperature due to suitable condition for growth of LA5 and BB12, which have optimum temperature for growth at 37 ◦C, the production of CLA is induced [40]. Based on the results of bibliographic studies, the highest CLA production by probiotics, especially *Lactobacillus* and *Bifidbacterium* strains, is done in the first 24 hours of incubation time, in exponential phase (logarithmic growth phase) and near (reaching) stationary phase [35, 41]. The findings are in good agreement with the results of this study. The main reason of bioconversion of linoleic acid to CLA by bacteria is unclear. However, some researchers proposed that it may be due to inhibitory effect of linoleic acid and detoxification mechanism of bacteria for growth [25, 42]. As primary metabolites, BACs, produced during the exponential phase, reach the maximum in the end of this phase or in the beginning of the stationary phase. Therefore, the maximum activity of bacteriocins can be usually observed at the time interval of 10 – 12 h of fermentation and decreased after that [38].

According to the similar results reported by Van Nieuwenhove et al. 2007, linoleic acid concentration had no significant effect on cell growth. Although the results demonstrated that the bioconversion process was extremely dependent on the free linoleic acid concentration, many authors unveiled the bacterial growth inhibition effect of linoleic acid with diverse tolerance for different strains. Recent studies reported that common probiotic strains were able to grow in low concentrations of linoleic acid [41]. Probiotic bacteria are

fastidious. However, their growth can be improved by the supplementation of the culture medium by amino acids (peptone, yeast extract, and beef extracts) and vitamins as well as compounds such as Sorbitan monooleate, sodium acetate and magnesium salts. Besides, due to the presence of Tween 80 in free linoleic acid solution, the interaction of free linoleic acid concentration and culture media was statistically significant [42]. Similar results were reported by Terán et al. 2015, and Macouzet et al. 2009, on the effect of linoleic acid concentration on CLA production. Terán et al. 2015 claimed that the highest percentages of conversion of linoleic acid to CLA were determined by adding linoleic acid concentrations lower than 500 $\mu g.mL^{-1}$; nevertheless, these percentages decreased with higher concentrations of linoleic acid. Alonso et al., 2003, reported that the highest amount of CLA was produced in 200 µL of free linoleic acid and decreased in 500 µL of free linoleic acid by *L. acidophilus* strains [42]. Khosravi et al. 2015, reported on that the linear effect of linoleic acid content on CLA production [25]. Similarly, Ye et al. 2013 reported CLA biosynthesize increased significantly by *L. acidophilus* LA5 after the addition of free linoleic acid into the medium. Considering all reported results, it seems as if, generally, there is a direct relationship between the conjugated linoleic acid bio-synthetization and the amount of linoleic acid in fermentation medium [40].

Yeast extract concentration is one of the best organic nitrogen sources with vibrant effect on probiotics growth and metabolites in combination by pH and temperature [39]. Generally, based on cell growth studies, an increase in nitrogen supplementation of the culture medium caused to increased biomass of bacterial culture. According to the literatures, the nitrogen source, generally improves biological changes in the fermentation bioprocess [39]. Alonso et al. 2003, investigated the effect yeast extract on the growth of lactic acid bacterial strains in the commercial CW, as a culture medium, and reported that in yeast extract supplemented culture media, cell density was found to be higher (about 1.8 $g.L^{-1}$), compared with non-supplemented medium (1.2 $g.L^{-1}$) [26]. Dey et al. 2012, showed that among the various nitrogen sources, yeast extract had the highest effect on biomass increase due to having high nitrogen content and including amino acids and vitamins. Moreover, Moon et al. 2014, reported that among the various complex sources of nitrogen, yeast extract is the best choice for the high biomass growth [24, 41]. Khosravi et al. 2015, claimed that the CLA productions were highly affected by yeast extract. Given the high buffering capacity of yeast extract with a complex nutritional source, consisting of amino acids, peptides, nucleotides, some carbohydrates, trace elements and Group-B vitamins, it is the most-frequently used nitrogen source for microbial growth. Some researchers reported on the linear effect of yeast extract concentration on CLA production [25]. Suresh Kumar et al. 2007, stated that EPSs production was improved by adding organic nitrogen sources into culture media [43]. According to Macedo et al. 2002, nitrogen source supplementation of whey permeate-based medium increased EPSs production by *L. rhamnosus* RW-9595M, which is in agreement with data presented in this study [44]. The results of this study agreed well with those of Deepak et al. 2016, demonstrating that the production of EPSs increased by increasing the concentration of yeast extract and the synthesis of EPSs was positively affected by the yeast extract [45].

Earlier researches reported that many compounds of culture media, such as proteins, could neutralize the negative effects of fatty acids on probiotics metabolism. Also bacterial growth in the milk-based media, like CW, was not affected in the presence of added linoleic acid and produced CLA. The results of this study are consistent with the findings of other authors who reported on the CLA production in the milk-based media [45]. The inhibitory effect of lactose high concentration on both biomass and BACs production has been reported by previous research [29]. According to the literatures, the CLA production is dependent on strains. Previous studies reported that LABs produced lower concentrations of CLA in compared with the Bifidobacteria. In current study, BB12 produced higher CLA concertation, which is similar with the results reported by Coakley et al. (2003) [46]. Terán et al. 2015 reported that the *B. animalis* subsp. *lactis* INL2 strain showed a high CLA production, depending on the concentration of the substrate present in the culture medium.

5. Conclusion

Initial pH, temperature and incubation time are very important environmental factors that affected the postbiotic metabolites co-production by fermentation bioprocess. Furthermore, yeast extract concentration is very important for biomass and growth associated metabolites such as three studied postbiotic metabolites. As well as supplementing culture media by free linoleic acid as a unique pre-source to CLA biosynthesis is very impotent and effective. Whereas, using food-grade low-cost CW and MP as culture media support cell growth and bioactive metabolites production, for high level of productivity supplementation is required. Both of investigated commercial probiotic bacteria were able to co-produce health-beneficial bioactive metabolites, however the results showed that capability of BB12 in this case was higher than LA5. Hence, probiotic bacteria be able to co-produce functional bioactive metabolites in supplemented dairy effluents. However, the concentrations of co-produced metabolites will be lower than the individually production of them by same probiotics. Further studies are needed to investigate other probiotic bacteria as well as carbon and nitrogen sources, minerals and stress condition to optimize co-production of postbiotic metabolites that led to higher productivity.

Acknowledgements

The authors are grateful to the Biotech center of Urmia University for allowing them to use that center's laboratory facilities and equipment during this research. The authors acknowledge Dr. Laya Rezazad Bari from laboratories of Saba college of higher education for her help in laboratory experiments.

Compliance with Ethical Standards

Authors declare that they have no conflict of interest.

References

[1] S. Pirsa, P. Abdolsattari, S. Peighambardoust, S. Fasihnia and S. Peighambardoust, Investigating microbial properties of traditional Iranian white cheese packed in active LDPE films incorporating metallic and organoclay nanoparticles. *Chem. Rev. Lett.*, 4 (2020) 168-174.

[2] K. Sharifi and S. Pirsa, Electrochemical sensors; Types and applications in the food industry. *Chem. Rev. Lett.*, 4 (2020) 192-201.

[3] S. Pirsa, F. Mohtarami and S. Kalantari, Preparation of biodegradable composite starch/tragacanth gum/Nanoclay film and study of its physicochemical and mechanical properties. *Chem. Rev. Lett.*, 3 (2020) 98-103.

[4] M. Taheri Sarhozaki, S. Aramideh, J. Akbarian amd S. Pirsa, Effects of ZnO nanoparticles and Kaolin in combination with NeemAzal-T/S against Bemisia tabaci and its parasitoid Eretmocerus mundus on cotton. *Chem. Rev. Lett.*, 3 (2020) 131-139.

[5] S. Pirsa and K. Aghbolagh Sharifi, A review of the applications of bioproteins in the preparation of biodegradable films and polymers. *J. Chem. Lett.*, 1(2) (2020) 47-58.

[6] S. Pirsa, E. Banafshechin, S. Amiri, A. Rahimirad and J. Ghafarzadeh, Detection of fraud of palm, sunflower, and corn oil in butter using HPLC profile of tocopherols and tocotrienols by response surface method. *J. Iran Chem. Soc.*, (2020) 1-11. doi.org/10.1007/s13738-020-02100-z

[7] S. Ghasemi, M.R. Bari, S. Pirsa and S. Amiri, Use of bacterial cellulose film modified by polypyrrole/TiO2-Ag nanocomposite for detecting and measuring the growth of pathogenic bacteria. *Carbohydr. Polym.*, 232 (2020) 115801.

[8] S. Amiry, M. Esmaiili and M. Alizadeh, Classification of adulterated honeys by multivariate analysis. *Food Chem.*, 224 (2017) 390-397.

[9] A. Gholam-Zhiyan, S. Amiri, M. Rezazadeh-Bari, S. Pirsa, Stability of *Bacillus coagulans* IBRC-M 10807 and *Lactobacillus plantarum* PTCC 1058 in Milk Proteins Concentrate (MPC)-Based Edible Film. *J. Package. Technol. Res.*, (2021) 1-12. doi.org/10.1007/s41783-021-00106-3

[10] S. Amiri, R.R. Mokarram, M.S. Khiabani, M.R. Bari and M. Alizadeh, Optimization of food-grade medium for co-production of bioactive substances by *Lactobacillus acidophilus* LA-5 for explaining pharmabiotic mechanisms of probiotic. *J. Food Sci. Technol.*, (2020) 1-12. doi.org/10.1007/s13197-020-04894-5

[11] O. Maleki, M.A. Khaledabad, S. Amiri, A.K. Asl and S. Makouie, Microencapsulation of *Lactobacillus rhamnosus* ATCC 7469 in whey protein isolate-crystalline nanocellulose-inulin composite enhanced gastrointestinal survivability. *LWT.*, 126 (2020) 109224.

[12] M. Rezazadeh-Bari, Y. Najafi-Darmian, M. Alizadeh and S. Amiri, Numerical optimization of probiotic Ayran production based on whey containing transglutaminase and *Aloe vera* gel. *J. Food Sci. Technol.*, 56 (2019) 3502-3512.

[13] Z.M. Moghanjougi, M.R. Bari, M.A. Khaledabad, H. Almasi and S. Amiri, Bio-preservation of white brined cheese (Feta) by using probiotic bacteria immobilized in bacterial cellulose: Optimization by response surface method and characterization. *LWT.*, 117 (2020) 108603.

[14] S. Amiri, M. Aghamirzaei, P. Mostashari, M. Sarbazi, S. Tizchang and H. Madahi, The impact of biotechnology on dairy industry. In Microbial Biotechnology in Food and Health, pp. 53-79. Academic Press (2020).

[15] D.K. Dahiya and A.K. Puniya, Isolation, molecular characterization and screening of indigenous lactobacilli for their abilities to produce bioactive conjugated linoleic acid (CLA). *J. Food Sci. Tech.*, 54 (2017) 792-801.

[16] V. Terán, P.L. Pizarro, M.F. Zacarías, G. Vinderola, R. Medina and C. Van Nieuwenhove, Production of conjugated dienoic and trienoic fatty acids by lactic acid bacteria and bifidobacteria. *J. Funct. Foods*, 19 (2015) 417-425.

[17] P. Kšonžeková, P. Bystrický, S. Vlčková, V. Pätoprstý, L. Pulzová, D. Mudroňová, ... and L. Tkáčiková, Exopolysaccharides of *Lactobacillus reuteri*: their influence on adherence of *E. coli* to epithelial cells and inflammatory response. *Carbohydr. Polym.*, 141 (2016) 10-19.

[18] W. Ren, Y. Xia, G. Wang, H. Zhang, S. Zhu and L. Ai, Bioactive exopolysaccharides from a *S. thermophilus* strain: Screening, purification and characterization. *Int. J. Biol. Macromol.*, 86 (2016) 402-407.

[19] S.C. Hsieh, J.M. Liu, X.H. Pua, Y. Ting, R.J. Hsu and K.C. Cheng, Optimization of *Lactobacillus acidophilus* cultivation using taro waste and evaluation of its biological activity. *Appl. Microbiol. Biotechnol.*, 100 (2016) 2629-2639.

[20] G. Ünlü, B. Nielsen and C. Ionita, Production of antilisterial bacteriocins from lactic acid bacteria in dairy-based media: a comparative study. *Probiotics Antimicrob. Proteins*, 7 (2015) 259-274.

[21] D. Drider, F. Bendali, K. Naghmouchi and M.L. Chikindas, Bacteriocins: not only antibacterial agents. *Probiotics Antimicrob. Proteins*, 8 (2016) 177-182.

[22] P. Wannun, S. Piwat and R. Teanpaisan, Purification, characterization, and optimum conditions of fermencin SD11, a bacteriocin produced by human orally *Lactobacillus fermentum*. *Appl. Biochem. Biotechnol.*, 179 (2016) 572-582.

[23] M. Pescuma, G.F. de Valdez and F. Mozzi, Whey-derived valuable products obtained by microbial fermentation. *Appl. Microbiol. Biotechnol.*, 99 (2015) 6183-6196.

[24] N. Rodríguez-Pazo, L. Vázquez-Araújo, N. Pérez-Rodríguez, S. Cortés-Diéguez and J.M. Domínguez, Cell-free supernatants obtained from fermentation of cheese whey hydrolyzates and phenylpyruvic acid by *Lactobacillus plantarum* as a source of antimicrobial compounds, bacteriocins, and natural aromas. *Appl. Biochem. Biotechnol.*, 171 (2013) 1042-1060.

[25] A. Khosravi, M. Safari, F. Khodaiyan and S.M.T. Gharibzahedi, Bioconversion enhancement of conjugated linoleic acid by *Lactobacillus plantarum* using the culture media manipulation and numerical optimization. *J. Food Sci. Technol.*, 52 (2015) 5781-5789.

[26] M. Haj-Mustafa, R. Abdi, M. Sheikh-Zeinoddin and S. Soleimanian-Zad, Statistical study on fermentation conditions in the optimization of exopolysaccharide production by *Lactobacillus rhamnosus* 519 in skimmed milk base media. *Biocat. Agric. Biotech.*, 4 (2015) 521-527.

[27] K. Zhou, Y.T. Zeng, X.F. Han and S.L. Liu, Modelling growth and bacteriocin production by *Lactobacillus plantarum* BC-25 in response to temperature and pH in batch fermentation. *Appl. Biochem. Biotechnol.*, 176 (2015) 1627-1637.

[28] S. Amiri, R.R. Mokarram, M.S. Khiabani, M.R. Bari and M.A. Khaledabad, *In situ* production of conjugated linoleic acid by *Bifidobacterium lactis* BB12 and *Lactobacillus acidophilus* LA5 in milk model medium. *LWT, 132* (2020) 109933.

[29] N.P. Guerra, M.L. Rua and L. Pastrana, Nutritional factors affecting the production of two bacteriocins from lactic acid bacteria on whey. *Int. J. Food Microb.*, 70 (2001) 267-281.

[30] L. Loghavi, S.K. Sastry and A.E. Yousef, Effect of moderate electric field on the metabolic activity and growth kinetics of *Lactobacillus acidophilus*. *Biotechnol. Bioeng.*, 98 (2007) 872-881.

[31] L.M. Rodríguez-Alcalá, T. Braga, F.X. Malcata, A. Gomes and J. Fontecha, Quantitative and qualitative determination of CLA produced by *Bifidobacterium* and lactic acid bacteria by combining spectrophotometric and Ag^{+}-HPLC techniques. *Food Chem.*, 125 (2011) 1373-1378.

[32] S. Amiri, R.R. Mokarram, M.S. Khiabani, M.R. Bari and M.A. Khaledabad, Exopolysaccharides production by *Lactobacillus acidophilus* LA5 and *Bifidobacterium animalis* subsp. lactis BB12: optimization of fermentation variables and characterization of structure and bioactivities. *Int. J. Biol. Macromol.*, 123 (2019) 752-765.

[33] S. Ye, T. Yu, H. Yang, L. Li, H. Wang, S. Xiao and J. Wang, Optimal culture conditions for producing conjugated linoleic acid in skim-milk by co-culture of different *Lactobacillus* strains. *Ann. Microbiol.*, 63 (2013) 707-717.

[34] C. Soto, *Lactobacillus plantarum* as source of conjugated linoleic acid: Effect of pH, incubation Temperature and inulin incorporation. *J. Biochem. Technol.*, 5 (2013) 649-653.

[35] M. Macouzet, B.H. Lee and N. Robert, Production of conjugated linoleic acid by probiotic *Lactobacillus acidophilus* La-5. *J. Appl. Microbiol.*, 106 (2009) 1886-1891.

[36] A. Laws, Y. Gu and V. Marshall, Biosynthesis, characterisation, and design of bacterial exopolysaccharides from lactic acid bacteria. *Biotechnol. Adv.*, 19 (2001) 597-625.

[37] B. Aslim, Z. Nur Yüksekdağ, Y. Beyatli and N. Mercan, Exopolysaccharide production by *Lactobacillus delbrueckii* subsp. bulgaricus and *Streptococcus thermophilus* strains under different growth conditions. *World J. Microb. Biotech.*, 21 (2005) 673–677.

[38] M. Zamfir, R. Callewaert, P.C. Cornea and L. Vuyst, Production kinetics of acidophilin 801, a bacteriocin produced by *Lactobacillus acidophilus* IBB 801. *FEMS Microbiol. Lett.*, 190 (2000) 305-308.

[39] P. Dey, J. Sikder, S. Roy and P. Pal, Fermentative lactic acid production from a renewable carbon source under response surface optimized conditions without alkali addition: a membrane-based green approach. *Clean Tech. Environ. Policy*, 14 (2012) 827-835.

[40] K. Khosravi-Darani, F.S. Reihani and R. Feili, Bioproduction of conjugated linoleic acid in yogurt by probiotic bacteria. *Int.*

[41] C.P. Van Nieuwenhove, R. Oliszewski, S.N. González and A.B. Perez Chaia, Conjugated linoleic acid conversion by dairy bacteria cultured in MRS broth and buffalo milk. *Lett. Appl. Microbiol.*, 44 (2007) 467-474.

[42] L. Alonso, E.P. Cuesta and S.E. Gilliland, Production of free conjugated linoleic acid by *Lactobacillus acidophilus* and *Lactobacillus casei* of human intestinal origin. *J. Dairy Sci.*, 86 (2003) 1941-1946.

[43] A. Suresh Kumar, K. Mody and B. Jha, Bacterial exopolysaccharides–a perception. *J. Basic Microbiol.*, 47 (2007) 103-117.

[44] M.C. Macedo, C. Lacroix and C.P. Champagne, Combined effects of temperature and medium composition on exopolysaccharide production by *Lactobacillus rhamnosus* RW-9595M in a whey permeate based medium. *Biotechnol. Prog.*, 18 (2002) 167–173.

[45] V. Deepak, S. Ram Kumar Pandian, S.D. Sivasubramaniam, H. Nellaiah and K. Sundar, Optimization of anticancer exopolysaccharide production from probiotic *Lactobacillus acidophilus* by response surface methodology. *Prep. Biochem. Biotech.*, 46 (2016) 288-297.

[46] M. Coakley, R.P. Ross, M. Nordgren, G. Fitzgerald, R. Devery and C. Stanton, Conjugated linoleic acid biosynthesis by human-derived *Bifidobacterium* species. *J. Appl. Microb.*, 94 (2003) 138–145.

How to Cite This Article

Saber Amiri; Mahmoud Rezazadeh Bari; Mohammad Alizadeh Khaledabad; Reza Rezaei Mokarram; Mahmoud Sowti Khiabani. "Co-production of parabiotic metabolites by Lactobacillus acidophilus LA5 and Bifidobacterium animalis subsp. lactis BB12 in dairy effluents". Chemical Review and Letters, 4,1, 2021, 66-76. doi: 10.22034/crl.2021.253739.1086