Diazinon Reduction In Apple Juice Using Probiotic Bacteria During Fermentation And Refrigerated Storage

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Abstract

The main objective of this work was to study the effects of probiotic strains, probiotic primary inoculated population, concentrations of spiked diazinon, physiology of probiotic bacteria, fermentation times and cold storage period in six consecutive stages on diazinon reduction in apple juice. Chemical properties (pH, total acidity, and sugar content), probiotic viability and diazinon reduction percent were monitored during fermentation and cold storage. Dispersive Solid Phase Extraction (dSPE) followed by gas chromatography-mass spectrometry was used to extract and measure diazinon concentration. Results showed that *Lactobacillus acidophilus* revealed the highest ability to reduce diazinon in apple juice after fermentation. Inoculation of *L. acidophilus* at 9 log CFU/mL, showed significantly higher diazinon reducing ability than 7 log CFU/mL. Ability of *L. acidophilus* to reduce the concentration of 1000 µg/L of spiked diazinon was significantly greater than 5000 µg/L. Heat-killed (dead) *L. acidophilus* bacteria reduced less diazinon content at the end of fermentation than viable bacteria. Furthermore, 72 h of fermentation was more effective in diazinon reduction. Level of diazinon in treatments containing *L. acidophilus* decreased significantly during cold storage period, so that it is completely disappeared at the end of storage (28 days) along with maintaining health-promoting properties of probiotic apple juice.

Introduction

Population rising and urbanization led to widespread utilization of different kinds of pesticides in agricultural practices during the last century (Mohammadi et al., 2020). However, the occurrence of pesticides residues in different food products is a concern for population health due to their detrimental activity in human body (Cengiz et al., 2006). Diazinon (O,O-diethyl-O-[2-isopropyl-6-methyl-4-pyrimidinyl] phosphoro thioate), is one of the most commonly used organophosphorus pesticides for plants' pest control via inhibition of acetyl cholinesterase (Mahmoudpoor Moteshaker et al., 2020; Shah & Iqbal, 2010). It is known as a genotoxic, mutatoxic, cytotoxic and neurotoxic compound which is classified as probable human carcinogen (Group 2A) by International Agency for Research on Cancer (IARC) (Mohammadi et al., 2020). Diazinon is generally used in agricultural crops including different types of fruits and vegetables like apples (Vera et al., 2020). Subsequently, its residues have been revealed in such products (Y. Zhang et al., 2010). After digestion of diazinon, it is converted to diaxozon by oxidative enzymes in the liver. Diaxozon is much more toxic than diazinon and causes generally the inhibition of acetyl cholinesterase in the body (Pordel et al., 2019).

Given the presence of diazinon residues in apple and high consumption rate of this crop and its products by general population, the determination and reduction of diazinon residues play a critical role in the food safety sector (Cengiz et al., 2006).

With the purpose of avoiding potential human exposure to contaminants such as pesticides via food consumption, there is seriously need to use helpful techniques for removal and degradation of pesticides' residues in different food products. Some physical and chemical methods have been investigated for the degradation of pesticides' residues including conventional methods like photolysis and hydrolysis, as

well as emerging techniques such as ozonation, hydrostatic pressure, ultrasound, cold plasma and ionizing radiation (Bai et al., 2010; Bhilwadikar et al., 2019). However, most of these mitigation techniques have some limitations especially high cost of investment and secondary contamination. To this end, bioremediation by microorganisms such as probiotics could be a promising method for pesticide's elimination (Mohammadi et al., 2020). Microbial degradation is a reliable and cost-effective technique with the potential for pesticides' removal from different food products (Islam et al., 2010).

Probiotics are defined as live microorganisms that, while consumed in adequate levels, present a health advantage on the host (Meybodi et al., 2020). Lactic acid bacteria and bifidobacteria are the most frequent types of bacteria used as probiotic (Kaur et al., 2016). It is well-known that probiotics are effective in prevention and/or treatment of numerous diseases and disorders vis. abdominal disorders, obesity, diabetes, lactose intolerance, hypertension, and different kinds of cancers via stabilizing the gut microflora, mounting micro- and macro-nutrients bioavailability and production of different bioactive substances like bacteriocins, enzymes and vitamins (Malganji et al., 2016; Nematollahi et al., 2016). Furthermore, it has been underlined that probiotic bacteria could degrade chemical contaminants including insecticides via binding to these xenobiotic compounds and producing enzymes (like phosphotriesterases, carboxylesterases, phosphatases, and organophosphate hydrolases) (Kaur et al., 2016).

Fermentation is an old process of food preparation that has been reported to be an efficient method for decreasing pesticides levels (Yousefi et al., 2021). There are several studies investigated the effects of probiotic fermentation on toxins' degradation like polycyclic aromatic hydrocarbons (PAH) in phosphate buffer (Yousefi et al., 2019), acrylamide in coffee beans (Choi et al., 2019), aflatoxin M1 (AFM1) in Doogh (Sarlak et al., 2017), patulin in apple juice (Zoghi et al., 2017), pirimiphos-methyl in wheat (T. Đorđević et al., 2013 a) and parathion in vinegar (Banna & Kawar, 1982). To best of our knowledge, the effect of probiotic bacteria on diazinon mitigation in apple juice has not been studied until now. Therefore, the aim of the present study is evaluating the effect of different factors on the ability of probiotic bacteria in removing diazinon in apple juice.

Material And Methods

2.1. Probiotics, concentrate and chemicals

The lyophilized probiotic strains including *Lactobacillus (L.) acidophilus*, *L. rhamnosus*, *L. casei* and *L. plantarum* were purchased from Parsilact Company, (Shiraz, Iran). Apple juice concentrate was acquired from Takdaneh Co. (Marand, Iran). Methanol, acetonitrile, sodium chloride, sodium hydroxide, magnesium sulphate, primary secondary amine and MRS agar were obtained from Merck Company (Germany). Fehling A and B solutions were purchased from Acros Organics Company (USA) and diazinon standard were obtained from Sigma-Aldrich (USA).

2.2. Preparation of diazinon working solution

Firstly, all 25 mg of diazinon standard solution was dissolved in 25 mL methanol to obtain a stock solution with concentration of 25000 mg/L. After that, working solution (1000 mg/L) was made by adding 400 µL of stock solution into 10 mL methanol. Then this working solution was used to obtain apple juice with concentration of 1 and 5 mg/L of diazinon in order to evaluate toxin reduction ability of probiotic bacteria. Diazinon standard solutions were stored at -18 ˚C.

2.3. Study design

This work was carried out based on the one-factor-at-a-time design in six steps. One independent factor (variable) was evaluated in each steps, and the sample with the best results was chose and applied in the next step. Therefore, the best level of independent variables in each steps was chosen for usage in the next step. The independent variables investigated were type of probiotic species (*L. rhamnosus*, *L. plantarum*, *L. acidophilus* and *L. casei*), primary probiotic population (7 and 9 log CFU/mL), diazinon concentration spiked (1 and 5 mg/L), dead and alive probiotic bacteria, fermentation times (24, 48 and 72 h, at 37 ˚C) and storage period at 4°C (0, 7, 14, 21 and 28 days).

2.4. Treatments and procedures

First of all, the apple juice concentrate (brix: 70°) was diluted to brix 15° using water. Then, the obtained apple juices were pasteurized for 20 min at 80°C. For the first stage, treatments were spiked with diazinon working solution until 1000 µg/L concentration was achieved. After that, the samples of apple juice were stirred thoroughly for 10 min to ensure diazinon dispersion. Then, the treatments were inoculated with one of the probiotic strain i.e. *L. rhamnosus*, *L. plantarum*, *L. acidophilus* and *L. casei*) at a primary concentration of 9 log CFU/mL. The treatments without probiotic and toxin addition were regarded as controls. The prepared treatments were incubated for 48 h at 37°C. The best probiotic strain regarding to viability and reduction ability in diazinon content was selected to investigate the next step regarding to compare the initial inoculation amount of 7 and 9 log CFU/mL. Therefore, the effect of initial inoculation rate was studied on the reduction of diazinon in apple juice samples. At the third step, the probiotic strain selected in the first step with the inoculation rate verified in the second step was added to apple juice samples in different spiked diazinon concentration (1000 and 5000 µg/L). Thus, in this stage, the best concentration of spiked diazinon was selected. At fourth step, the probiotic strain selected in the first step was added to apple juice samples in the inoculation rate determined in the second step and initial concentration of spiked diazinon observed in the third step prior to heat-treatment (90°C for 15 min) to kill any viable probiotic bacteria to compare alive and dead cells in diazinon reduction percent. In the fifth step, the probiotic strain selected in the first step with the inoculation rate determined in the second step and diazinon concentration observed in the third stage and live or dead probiotic strain in fourth step was added to apple juice samples for evaluation different fermentation times (24, 48 and 72 h) in diazinon reduction percent. In the sixth step, the probiotic strain selected in the first step with the inoculation rate determined in the second step, diazinon concentration observed in the third step, live or dead probiotic bacteria determined in fourth step and the optimal fermentation time selected in the fifth step was added to apple juice samples to evaluate diazinon reduction rate during cold storage at 4˚ C in the intervals of 0, 7, 14, 21 and 28 days. The chemical properties, viability of probiotic bacteria and reduction level of

diazinon concentration in the apple juice samples were measured during fermentation and cold storage (for six stages). All treatments were investigated in triplicate.

2.5. Chemical analysis

2.5.1. pH value

pH was measured by a digital pH meter (Metrohm, Switzerland) with direct insertion of the electrode into the apple juice samples at room temperature (Güney & Güngörmüşler, 2020).

2.5.2. Total acidity

To measure total acidity of apple juices, 20 mL of sample mixed with 50 mL of distilled water was titrated by sodium hydroxide (0.1 N) to achieve pH 8.1 (Cengiz et al., 2006). Total acidity was calculated using following equation:

$$A = V \times \frac{0.0067 \times 100}{m} \qquad \text{(Equation 1)}$$

Where, V = volume of consumed sodium hydroxide, m = apple juice weight in gram and A = total acidity in grams of malic acid per 100 grams of fruit juice.

2.4.3. Sugar content

The sugar concentration (total and reducing sugars) in apple juice samples were measured using Fehling's method (De Carvalho et al., 2007). This method is based on the fact that reducing sugars reduces the copper present in Fehling-A solution to a brick-red insoluble cuprous oxide. In the Fehling' method we used two different solutions i.e. Fehling A (copper sulfate in distilled water) and Fehling B (potassium sodium tartrate and sodium hydroxide in distilled water). 5 milliliters of each Fehling-A and B were added into a 250-mL Erlenmeyer. The solution was blended and 20 mL of distilled water and four or five boiling glass beads were added to the Erlenmeyer. The Erlenmeyer was then heated to boiling point and a few drops of methylene blue (as indicator) were added. Drop-wise addition of the apple juice sample inside the burette was performed till the blue color vanishes to a brick-red color.

The reducing sugar content was calculated according to the Eq. 2:

$$n = \frac{F \times 100 \times 100}{V \times 25} \qquad \text{(Equation 2)}$$

Where F = Fehling factor; V = Consumption volume of apple juice solution in mL; n = Reducing sugars (sugar before hydrolysis) in g per 100 g.

The total sugar content was calculated using Eq. 3 after acid hydrolysis of apple juice sample.

$$n = \frac{F \times 100 \times 100 \times 100}{V \times 25 \times 25} \qquad \text{(Equation 3)}$$

2.5. Microbiological analysis

The number of viable probiotics (CFU/mL) were ascertained using plate count methodology (MRS-bile agar medium (enclosing 0.15% bile salts), incubated at 37°C, facultative aerobically) according to Mortazavian et al. method (Mortazavian et al., 2007).

2.6. Determination of diazinon content

2.6.1. Sample preparation

The clean-up and extraction stages were done based on dispersive Solid Phase Extraction (dSPE)-QuEChERS-method suggested by Anastassiades and Lehotay with slight modification (Anastassiades et al., 2003). Briefly, 20 mL of each apple juice sample was weighed in the centrifuge tube (50 mL). In order to precipitate the suspended solids present in the juices, the samples was centrifuged at 2800×g for 3 min. Then, 10 mL of the upper clear aqueous phase was separated, and moved to another tube. After that, 10 mL of acetonitrile was added and then was shaken strongly for 1 min (first extraction stage). Then, 4 g magnesium sulphate ($MgSO_4$), 1 g sodium chloride (NaCl) and 1 g trisodium citrate 2 hydrate ($C_6H_5NaO_7$. 2 H_2O) were added to the solution and were shaken a few seconds after salt addition. This solution was shaken completely for 1 min and then was centrifuged at 2800×g for 3 min (second extraction stage). Then, 2 mL of the supernatant was added to a polypropylene tube containing 0.5 g Primary secondary amine (PSA) and 0.15 g $MgSO_4$ to extract diazinon using DSPE method. After shaking for 2 min and centrifuging for 5 min at 2800×g, 1 mL of obtained extracted solution was mixed with 10 µL of acid formic (5%) in acetonitrile in a glass vial for acidification. 1.5 µL of extracted solution was injected to Gas Chromatography-Mass Spectrometry (GC-MS) for determination of diazinon residual in the apple juice samples.

2. 6. 2. GC analysis

A 7890 B GC system from Agilent Technologies (Palo Alto, CA, USA) couple with a triple-axis detector equipped with a split/splitless injector is used for GC-MS technique. This system was coupled with an Agilent (7977 A MSD) network mass selective detector. An HP-5 capillary column, with length, interior diameter and film thickness of 30 m× 0.32 mm and 0.25 µm, respectively was used to separate chemical substances. The temperature program of GC was: isothermal at 100°C for 5 min, risen to 185°C at 5°C/min, and again isothermal for 1 min, risen to 290°C at 15°C/min, and again isothermal for 15 min. Helium gas was applied as a carrier gas at a stable flow rate of 1 mL/min. The injection volume was also 1 mL/min. Purge flow to split vent was adjusted at 40 mL/min. One microliter of the bottom phase of the solution was introduced in a splitless manner. The retention time for diazinon was 9.25 min.

2.6.3. Calculations

Calculations were made according to the Eq. 4:

$$\text{Total diazinon concentration } (\%) = \frac{1000 \text{ or } 5000\ \mu g/L - Wm}{1000 \text{ or } 5000\ \mu g/L} \times 100 \qquad \text{(Equation 4)}$$

Where, Wm = amount of diazinon in the test sample in µg/L;

Probiotic affected diazinon reduction (%) was calculated by subtraction of total diazinon concentration (%) in probiotic treatment from that in control sample.

2.6.4. Figure of merits

The explained method (dSPE-GC-MS) was validated by linearity, recovery, repeatability (RSD %), limit of detection (LOD), and limit of quantitation (LOQ) under experimental conditions. The calibration curve presented the linearity at the range of 10–1000 µg/L. The correlation coefficient (R^2) calculated about 0.999. The LOD and LOQ were calculated by multiplying 3 and 10 at applicable standard deviation of analysis's repetitions (0.998). Thus, LOD and LOQ were calculated as 2.99 µg/L and 9.98 µg/L, respectively. The repeatability (RSDs), calculated based on comparative peak areas of seven replicate extraction methods from apple juice sample and this was calculated as 0.29 %. The recovery percentage of diazinon was determined to be 98% by calculating the amount of diazinon standard added to the samples (100 µg/L) with the level after the dSPE method.

2.7. Statistical analysis

All treatments were investigated in triplicate in the present study. One-way analysis of variance (ANOVA) was used to evaluate statistical difference between means of the treatments for chemical and properties, viability of probiotics as well as the diazinon content for each stages of study using SPSS software (version 18) at significance level of 0.05.

Results And Discussion

The chemical properties of prepared apple juice were determined as follow: pH = 4.08, total acidity = 0.28 g mallic acid/100 g, reducing sugar content = 12 g/100 g, total sugar content = 14.09 g/100 g, Brix = 15 and diazinon concentration < 10 µg/L. The results of effect of different parameters on chemical properties (pH, total acidity, reducing sugar and total sugar), diazinon content and microbiological properties (viability of probiotic bacteria) are discussed in the following.

3.1. Effect of probiotic strain's type on diazinon reduction (first stage)

The effect of different probiotic strains i.e. *L. rhamnosus*, *L. plantarum*, *L. acidophilus* and *L. casei*) at a primary concentration of 9 log CFU/mL and presence of 1000 µg/L of spiked diazinon on chemical and microbiological properties of apple juice samples was shown in Table 1 after 48 h fermentation at 37°C.

Table 1

Chemical and microbial changes of apple juice samples in the first stage*

Treatment**	pH	Total acidity (g/100 g)	Reducing sugar content (g/100 g)	Total sugar content (g/100 g)	Viability of probiotic (CFU/mL)	Diazinon reduction (%)
B	4.06 ± 0.07^{a}	0.28 ± 0.22^{a}	11.86 ± 0.01^{a}	13.80 ± 0.15^{a}	-	-
B-1	4.06 ± 0.07 ^{a}	0.28 ± 0.23^{a}	11.86 ± 0.01^{a}	13.86 ± 0.15^{a}	-	15.46^{a}
A-9-1	3.94 ± 0.02^{bd}	0.30 ± 0.28^{a}	11.42 ± 0.03^{b}	12.89 ± 0.18^{b}	10.22 ± 0.03^{a}	59.80^{b}
A-9	3.86 ± 0.01^{c}	0.44 ± 0.40^{b}	10.64 ± 0.05^{c}	12.06 ± 0.14^{c}	10.83 ± 0.02^{b}	-
R-9-1	3.96 ± 0.01^{d}	0.29 ± 0.22^{a}	11.61 ± 0.02^{d}	13.07 ± 0.32^{d}	10.20 ± 0.01^{a}	56.97^{c}
R-9	3.88 ± 0.01^{cb}	0.43 ± 0.42^{b}	11.02 ± 0.02^{e}	12.12 ± 0.12^{c}	10.80 ± 0.01^{b}	-
C-9-1	3.98 ± 0.08^{d}	0.29 ± 0.32^{a}	11.61 ± 0.03^{d}	13.29 ± 0.25^{e}	10.17 ± 0.06^{a}	56.77^{d}
C-9	3.89 ± 0.01^{bc}	0.42 ± 0.32^{b}	11.07 ± 0.02^{e}	12.50 ± 0.38^{f}	10.79 ± 0.01^{b}	-
P-9-1	3.99 ± 0.01^{bd}	0.29 ± 0.23^{a}	11.70 ± 0.04^{d}	13.50 ± 0.13^{g}	10.10 ± 0.05^{c}	56.37^{e}
P-9	3.90 ± 0.01^{c}	0.41 ± 0.31^{b}	11.22 ± 0.02^{f}	12.70 ± 0.12^{h}	10.67 ± 0.02^{d}	-

*Each value in the table is the mean ± standard deviation (SD) of three trials. Different letters in each column indicate a statistically significant difference ($P < 0.05$).

** B: control sample, 1: 1000 µg/L of spiked diazinon, A: *L. acidophilus*, R: *L. rhamnosus*, C: *L. casei* and P: *L. plantarum*, 9: Primary probiotic population (9 log CFU/mL).

As presented in this table, the pH of all treatments decreased from 4.08 to 3.86–4.06 after 48 h fermentation. These decline were significant ($p < 0.05$) for all treatments except control samples (B and B-

1) which contained no probiotic bacteria. The lowest pH content (3.86) was showed in samples containing *L. acidophilus* (A-9). The total acidity of all treatments increased significantly ($p < 0.05$) after 48 h fermentation from 0.28 to 0.29–0.44 g mallic acid/100 g apple juice with the exception of control samples without probiotic addition (B and B-1). The highest total acidity (0.44 g mallic acid/100 g) was showed in samples containing *L. acidophilus* (A-9). The reducing and total sugar content of all samples also decreased after fermentation. The lowest decline in sugar concentration was occurred in control samples (B and B-1), while the highest decline in reducing and total sugar content were observed in samples containing *L. acidophilus* (A-9) which determined 10.64 and 12.06 g/100 g apple juice, respectively.

Probiotic bacteria are able not only to survive but to use apple juice for their cell synthesis, as determined by a decrease in total and reducing sugar concentrations as well as pH value, and increase in acidity. Similar to previous studies concerning probiotic apple juice, probiotics may have consumed carbohydrates and formed small concentrations of organic acids thus lowering the pH and increasing the total acidity of the juice during fermentation and storage (Ding & Shah, 2008; Pimentel et al., 2015). It is worthy to note that different types of probiotic have different activity rate (Nematollahi et al., 2016). It is clear that *L. acidophilus* was observed to utilize the sugar and subsequently change pH and acidity at a faster rate than other strains.

The probiotic viability of samples containing different probiotic species increased significantly from 9 to 10.10-10.83 log CFU/mL during fermentation period. The highest and lowest probiotic viability were observed in samples containing *L. acidophilus* (A-9) and *L. plantarum* in the presence of added diazinon (P-9-1). It is worthy to mention that the probiotic viability in all samples with addition of diazinon was lower than samples without spiked diazinon. For example, the probiotic viability in A-9 and A-9-1 treatments were 10.83 and 10.22 log CFU/mL, respectively.

The diazinon reduction percent in control sample (B-1) was the lowest (15.46%), while the highest content was observed in samples containing *L. acidophilus* (A-9-1) which was 75.26 % which means the *L. acidophilus* decreased spiked diazinon as 59.80%. The diazinon reduction percent of other strains had low differences but significant (56.37–56.97 %). Yousefi et al. (2019) and Sarlak et al. (2016) also reported that *L. acidophilus* have the highest binding capacity to PAH and AFM1, respectively (Sarlak et al., 2017; Yousefi et al., 2019). Thus, *L. acidophilus* is the most effective probiotic strain in diazinon reduction in apple juice during fermentation and was chose for the next stage.

3.2. Effect of primary probiotic population on diazinon reduction (second stage)

In this stage, the effect of initial inoculated population (9 and 7 log CFU/mL) of selected probiotic strain from the previous stage (*L. acidophilus*) on diazinon reduction percent was evaluated. Table 2 shows the chemical and microbial changes of apple juice samples after 48 h fermentation at 37°C.

Table 2

Chemical and microbial changes of apple juice samples in the second stage*

Treatment**	pH	Total acidity (g/100 g)	Reducing sugar content (g/100 g)	Total sugar content (g/100 g)	Viability of probiotic (CFU/mL)	Diazinon reduction (%)
B	4.06 ± 0.01^{a}	0.28 ± 0.32^{a}	11.86 ± 0.02^{ab}	13.82 ± 0.05^{a}	-	-
B-1	4.06 ± 0.01^{a}	0.28 ± 0.20^{a}	11.93 ± 0.01^{a}	13.82 ± 0.13^{a}	-	15.53^{a}
A-9-1	3.95 ± 0.01^{b}	0.30 ± 0.30^{a}	11.42 ± 0.08^{c}	12.91 ± 0.19^{b}	10.20 ± 0.01^{a}	59.40^{b}
A-9	3.86 ± 0.07^{c}	0.43 ± 0.32^{b}	10.53 ± 0.05^{d}	11.98 ± 0.10^{c}	10.83 ± 0.05^{b}	-
A-7-1	3.98 ± 0.01^{b}	0.29 ± 0.23^{a}	11.80 ± 0.01^{b}	13.71 ± 0.10^{d}	7.35 ± 0.07^{c}	44.40^{c}
A-7	3.92 ± 0.01^{b}	0.34 ± 0.32^{a}	11.55 ± 0.03^{d}	12.83 ± 0.10^{e}	7.77 ± 0.04^{d}	-

*Each value in the table is the mean ± standard deviation (SD) of three trials. Different letters in each column indicate a statistically significant difference ($P < 0.05$).

** B: control sample, 1: 1000 µg/L of spiked diazinon, A: *L. acidophilus*, 9 or 7: Primary probiotic population (9 or 7 log CFU/mL).

As shown in Table 2, the pH of all samples reduced after 48 h fermentation which this decline was significant ($p < 0.05$) for all samples except control samples (B and B-1). The lowest pH content (3.86) observed in samples containing 9 log CFU/mL of *L. acidophilus* (A-9). The total acidity of all samples increased from 0.28 to 0.29–0.43 g mallic acid/100 g apple juice except in control samples (B and B-1) which did not change during fermentation. The highest total acidity was also observed in samples containing 9 log CFU/mL of *L. acidophilus* (A-9). The reducing and total sugar content of all samples decreased to 10.53–11.86 and 11.98–13.82 g/100 g apple juice, respectively. The highest sugar consumption was also reported for samples containing 9 log CFU/mL of *L. acidophilus* (A-9).

As it is obvious the chemical changes of samples containing 9 log CFU/mL of *L. acidophilus* (A-9) was significantly higher than that in A-7 indicating higher activity of probiotics during fermentation in higher primary population count.

The viability of *L. acidophilus* increased more than one cycle in the samples containing 9 log CFU/mL and reached to 10.20 and 10.83 log CFU/mL after 48 h fermentation, while this increment was lower than one cycle in the samples containing 7 log CFU/mL and reached to 7.35 and 7.77 log CFU/mL. The diazinon reduction percent in samples containing 9 log CFU/mL of *L. acidophilus* was significantly higher than that in samples containing 7 log CFU/mL which were 59.40 and 44.40 %, respectively due to higher activity of *L. acidophilus* in higher primary population.

L. aciduphilus degraded diazinon by using it directly as a source of carbon, nitrogen and phosphorus, or by creating diazinon-degrading enzymes. Inoculum primary amount plat a significant role in pesticide biodegradation. Lower initial inoculum rate could lead to a small number of the probiotics contributing in chemical degradation (Zhou & Zhao, 2015). Similar result was also observed in the degradation of organophosphorus pesticides during fermentation of pickled Chinese cabbage (Lu et al., 2013), degradation of AFM1 in Doogh (Sarlak et al., 2017), in vitro removal of PAH (Yousefi et al., 2019) and AFM1 (Kabak & Var, 2008) from phosphate-buffer by probiotic bacteria. To this end the primary population rate of 9 log CFU/mL was selected for the next stage.

3.3. Effect of diazinon concentration spiked on its reduction (third stage)

In this stage, the effect of different concentrations of spiked diazinon (1000 and 5000 µg/L) on its reduction by 9 log CFU/mL of *L. acidophilus* in apple juice was evaluated during 48 h fermentation at 37°C. Table 3 shows the chemical and microbial changes of apple juice samples after 48 h fermentation. Similar to previous stages the pH, total and reducing sugar concentrations decreased, while the total acidity of all samples increased after fermentation.

Table 3

Chemical and microbial changes of apple juice samples in the third stage*

Treatment**	pH	Total acidity (g/100 g)	Reducing sugar content (g/100 g)	Total sugar content (g/100 g)	Viability of probiotic (CFU/mL)	Diazinon reduction (%)
B	4.06 ± 0.07^{a}	0.28 ± 0.32^{a}	11.86 ± 0.01^{a}	13.86 ± 0.10^{a}	-	-
B-1	4.06 ± 0.01^{a}	0.28 ± 0.26^{a}	11.93 ± 0.01^{b}	13.86 ± 0.10^{a}	-	17.50^{a}
B-5	4.06 ± 0.01^{a}	0.28 ± 0.32^{a}	12.00 ± 0.01^{b}	14.02 ± 0.15^{b}	-	35.26^{b}
A-9-1	3.94 ± 0.01^{b}	0.30 ± 0.28^{a}	11.42 ± 0.02^{c}	12.93 ± 0.16^{c}	10.20 ± 0.09^{a}	57.53^{c}
A-9	3.86 ± 0.09^{c}	0.44 ± 0.60^{b}	10.90 ± 0.02^{d}	11.95 ± 0.22^{d}	10.82 ± 0.01^{b}	-
A-9-5	3.98 ± 0.06^{b}	0.29 ± 0.39^{a}	11.67 ± 0.02^{e}	13.37 ± 0.30^{e}	9.88 ± 0.04^{c}	23.63^{d}

*Each value in the table is the mean ± standard deviation (SD) of three trials. Different letters in each column indicate a statistically significant difference ($P < 0.05$)

** B: control sample, 1 or 5: 1000 or 5000 µg/L of spiked diazinon, A: *L. acidophilus*, 9: Primary probiotic population (9 log CFU/mL).

As depicted in Table 3, the presence of diazinon in apple juice samples containing probiotic bacteria (A-9-1 and A-9-5) led to decreasing in probiotic activity and viability, significantly ($p < 0.05$). For instance, the decline rate of pH, reducing and total sugar content in treatments containing *L. acidophilus* and 5000 µg/L of diazinon (A-9-5) was lower than that in samples containing 1000 µg/L of diazinon (A-9-1) and *L. acidophilus* without spiked diazinon (A-9). Furthermore, the viability of *L. acidophilus* in A-9, A-9-1 and A-9-5 was determined 10.82, 10.20 and 9.88 log CFU/mL, respectively which indicated that the presence of diazinon has negative effect on probiotic viability and subsequently their activity during fermentation. Thus, the ability of *L. acidophilus* to degrade the diazinon decreases with higher concentration of spiked diazinon. As shown in Table 3, the diazinon reduction percent by probiotic bacteria in samples containing 5000 µg/L of spiked diazinon (A-9-5) was lower than those containing 1000 µg/L of diazinon (A-9-1) which were 57.53 and 23.63 % which is in good agreement with Dordevic et al. (2013) (T. Đorđević et al., 2013 a) and Yousefi et al. (2019) (Yousefi et al., 2019) studies. In fact, the presence of diazinon could be effective in probiotic viability and activity (acid and other compounds' production) which was also

confirmed by Ayana et al. (2011) in a study done in yogurt in the presence of some pesticides (Ayana et al., 2011). Dordevic et al. (2013) also reported that pirimiphos-methyl (a pesticide) could just inhibit the growth of *L. plantarom* in concentrations higher than 5 mg/kg during wheat fermentation (T. Đorđević et al., 2013 a). In another study it is also reported that during the probiotic fermentation, bifenthrin reduction within wheat fortified with 500 µg/kg was 42%, although significantly lower in samples spiked with 2.5 µg/kg, maximum 18% (T. M. Đorđević et al., 2013 b). Thus, the spiked diazinon concentration of 1000 µg/L was selected for next stage.

3.4. Effect of alive and dead probiotic bacteria on diazinon reduction (fourth stage)

In this stage the effect of alive and dead *L. acidophilus* on diazinon degradation was compared during 48 h fermentation. Table 4 shows the chemical and microbial changes of apple juice samples after 48 h fermentation at 37°C.

Table 4

Chemical and microbial changes of apple juice samples in the fourth stage*

Treatment**	pH	Total acidity (g/100 g)	Reducing sugar content (g/100 g)	Total sugar content (g/100 g)	Viability of probiotic (CFU/mL)	Diazinon reduction (%)
B	4.06 ± 0.01^{a}	0.28 ± 0.26^{a}	11.86 ± 0.06^{a}	13.82 ± 0.06^{a}	-	-
B-1	4.06 ± 0.01^{a}	0.28 ± 0.25^{a}	12.13 ± 0.06^{b}	13.86 ± 0.06^{a}	-	16.26^{a}
A-9-1	3.94 ± 0.01^{b}	0.30 ± 0.35^{ab}	11.36 ± 0.05^{c}	12.89 ± 0.05^{b}	10.20 ± 0.05^{a}	57.97^{b}
A-9	3.86 ± 0.01^{c}	0.44 ± 0.37^{c}	10.96 ± 0.02^{d}	12.13 ± 0.02^{c}	10.83 ± 0.01^{b}	-
A-9D-1	3.99 ± 0.01^{b}	0.29 ± 0.42^{ab}	11.67 ± 0.02^{e}	13.29 ± 0.02^{d}	-	27.74^{c}
A-9D	3.93 ± 0.02^{b}	0.35 ± 0.26^{b}	11.61 ± 0.02^{e}	13.57 ± 0.02^{e}	-	-

*Each value in the table is the mean ± standard deviation (SD) of three trials. Different letters in each column indicate a statistically significant difference ($P < 0.05$).

** B: control sample, 1: 1000 µg/L of spiked diazinon, A: *L. acidophilus*, 9: Primary probiotic population (9 log CFU/mL), D: dead probiotic bacteria (killed by heat).

As depicted in Table 4, the apple juice treatments including heat-killed *L. acidophilus* (A-9D and A-9D-1) had slower chemical changes than samples containing alive probiotic bacteria i.e. A-9 and A-9-1, after 48 h fermentation. Ding and Shah (2008) reported that the dead probiotic bacteria could also release enzymes for hydrolyzing sugars in the fruit juice, consequently increasing the acidity and decreasing the pH and sugar content although it is lower than live cells (Ding & Shah, 2008) which is in good consistence with our study. Microbiological analysis of A-9 and A-9D-1 samples indicated the absence of any viable probiotic bacteria (data not shown). These results show that bacterial viability is a prerequisite for diazinon removal via enzyme production. However, the reduction percent in diazinon concentration by dead probiotic bacteria (27.74 %) revealed that probiotic bacteria could also reduce diazinon via binding capacity to this pesticide.

Generally, pesticide degradation is done either via degradation or absorption mechanisms. Several groups of microbial enzymes, including carboxylesterases, phosphatases, phosphotriesterases, organophosphorus hydrolases, may facilitate degradation of organophosphate pesticides like diazinon via the hydrolysis of phosphoric acid esters. Both acid and alkaline phosphatases may degrade organophosphate pesticides by hydrolyzing the C-O-P linkage of a wide variety of phosphate esters (Mohammadi et al., 2020; Y.-H. Zhang et al., 2014). Regarding to higher diazinon reduction percent in A-9-1 samples (57.97 %) than that in A-9D-1 samples (27.74 %), it could be concluded that 27.74 % and 30.23 % of diazinon reduction percent may be attributed to absorption and degradation mechanisms, respectively.

3.5. Effect of fermentation times on diazinon reduction (fifth stage)

Fermentation is a promising biological process, and decrease in pesticide residues through this procedure has been continuously investigated in several food commodities and buffer systems (Bo et al., 2011; T. Đorđević et al., 2013 a; T. M. Đorđević et al., 2013 b; KUMRAL et al., 2020). Some microorganisms are capable of utilizing pesticides as a carbon and phosphorus source and it has been observed that enzymes isolated from them are able to detoxify diazinon. They may also decrease diazinon through binding pesticides via agents existed in their cell wall (Cho et al., 2009; T. Đorđević et al., 2013 a; T. M. Đorđević et al., 2013 b; Mohammadi et al., 2020; Wang et al., 2016).

In this stage the effect of different fermentation times on diazinon reduction percent was investigated. Table 5 shows the chemical and microbial changes of apple juice samples during 72 h fermentation at 37°C with 24 h intervals.

Table 5

Chemical and microbial changes of apple juice samples in the fifth stage*

Parameter	Treatment	Fermentation times (h)		
		24	48	72
pH	B	4.08 ± 0.08^{aA}	4.06 ± 0.01^{aA}	4.05 ± 0.01^{aA}
	B-1	4.07 ± 0.08^{aA}	4.06 ± 0.01^{aA}	4.06 ± 0.01^{aA}
	A-9-1	4.02 ± 0.08^{abA}	3.94 ± 0.02^{bB}	3.90 ± 0.02^{cB}
	A-9	3.97 ± 0.02^{bA}	3.86 ± 0.08^{cB}	3.84 ± 0.01^{cB}
Total acidity (g/100 g)	B	0.28 ± 0.20^{aA}	0.28 ± 0.46^{aA}	0.28 ± 0.32^{aA}
	B-1	0.28 ± 0.25^{aA}	0.28 ± 0.26^{aA}	0.29 ± 0.38^{aA}
	A-9-1	0.29 ± 0.27^{abA}	0.30 ± 0.38^{aA}	0.31 ± 0.32^{aA}
	A-9	0.35 ± 0.32^{bA}	0.44 ± 0.50^{bB}	0.45 ± 0.32^{bC}
Reducing sugar content (g/100 g)	B	11.86 ± 0.01^{aA}	11.86 ± 0.02^{aA}	11.80 ± 0.01^{aA}
	B-1	12.00 ± 0.05^{aA}	11.93 ± 0.03^{aA}	11.86 ± 0.02^{aB}
	A-9-1	11.67 ± 0.02^{bA}	11.36 ± 0.02^{bB}	11.25 ± 0.02^{bC}
	A-9	11.36 ± 0.02^{cA}	10.64 ± 0.20^{cB}	9.47 ± 0.10^{cC}
Total sugar content (g/100 g)	B	13.82 ± 0.05^{aA}	13.75 ± 0.02^{aA}	13.64 ± 0.05^{aB}
	B-1	13.98 ± 0.07^{bA}	13.82 ± 0.15^{aB}	13.75 ± 0.02^{aB}
	A-9-1	13.43 ± 0.07^{cA}	12.91 ± 0.06^{bB}	12.80 ± 0.05^{bC}
	A-9	12.87 ± 0.07^{dA}	11.98 ± 0.30^{bB}	11.44 ± 0.18^{cC}
Viability of probiotic (CFU/mL)	B	-	-	-
	B-1	-	-	-
	A-9-1	9.44 ± 0.09^{aA}	10.21 ± 0.06^{aB}	10.23 ± 0.05^{aB}

*Each value in the table is the mean ± standard deviation (SD) of three trials. Different lower and upper letters in each column and row, respectively indicate a statistically significant difference ($P < 0.05$).

** B: control sample, 1: 1000 µg/L of spiked diazinon, A: *L. acidophilus*, 9: Primary probiotic population (9 log CFU/mL).

Parameter	Treatment	Fermentation times (h)		
		24	48	72
	A-9	9.82 ± 0.08^{bA}	10.82 ± 0.02^{bB}	10.85 ± 0.04^{bB}
Diazinon reduction (%)	B	-	-	-
	B-1	5.16^{aA}	15.36^{aB}	20.13^{aC}
	A-9-1	22.57^{bA}	58.70^{bB}	64.37^{bC}
	A-9	-	-	-
*Each value in the table is the mean ± standard deviation (SD) of three trials. Different lower and upper letters in each column and row, respectively indicate a statistically significant difference ($P < 0.05$).				
** B: control sample, 1: 1000 µg/L of spiked diazinon, A: *L. acidophilus*, 9: Primary probiotic population (9 log CFU/mL).				

As shown in Table 5, the pH, reducing sugar content and total sugar content decreased with increasing fermentation time while, probiotic viability and diazinon reduction percent increased.

The pH value of samples containing *L. acidophilus* (A-9) and samples containing *L. acidophilus* and 1000 µg/L spiked diazinon (A-9-1) decreased from 4.08 to 3.84 and 3.90, respectively which are significantly ($p < 0.05$) lower than control samples (B and B-1) in all fermentation times. The total acidity of A-9 and A-9-1 treatments increased from 0.28 g mallic acid/100 g apple juice to 0.45 and 0.31, respectively which are significantly ($p < 0.05$) higher than control samples (B and B-1) in all fermentation times. The reducing sugar content of A-9 and A-9-1 treatments decreased from 12 g /100 g apple juice to 9.47 and 11.25, respectively which are significantly ($p < 0.05$) lower than control samples (B and B-1) in all fermentation times. The total sugar content of A-9 and A-9-1 treatments also decreased from 14.09 g /100 g apple juice to 11.44 and 12.80, respectively which are significantly ($p < 0.05$) lower than control samples (B and B-1) in all fermentation times. In the case of probiotic viability, it is observed that the viability of *L. acidophilus* in A-9 and A-9-1 treatments increased from 9 log CFU/mL to 10.85 and 10.23 log CFU/mL, respectively. The diazinon reduction percent by *L. acidophilus* was calculated 22.57, 58.70 and 64.37 % after 24, 48 and 72 h fermentation, respectively.

As it could be detected in Table 5, the majority of noted changes observed in probiotic population, pH, total acidity, sugar consumption and diazinon reduction percent, happened in the period between hours 24 to 48 of the fermentation. Further extension of the fermentation process (from 48 to 72 h) the changes were slower but significant ($p < 0.05$) except in the case of pH value. It is obvious that with increasing fermentation time, diazinon reduction percent occurred by *L. acidophilus* increased significantly ($p < 0.05$). Thus, 72 h fermentation time was chose for the final stage.

In a similar study, Cho et al. (2009) reported that with increasing fermentation time for kimchi production, the degradation content of organophosphorus insecticide chlorpyrifos increased significantly. They

reported that lactic acid bacteria degraded 83.3% of chlorpyrifos until 72 h and degraded it completely by 216 h (Cho et al., 2009).

3.6. Effect of refrigerated storage on diazinon reduction (sixth stage)

In the final stage, the effect of 28 days of cold storage on diazinon reduction percent of fermented apple juice samples was investigated. Table 6 shows the chemical and microbial changes of fermented apple juice samples during 28 days of cold storage at 4°C with 7 days intervals.

Table 6

Chemical and microbial changes of apple juice samples in the sixth stage*

Parameter	Treatment**	Refrigerated storage times (day)				
		0	7	14	21	28
pH	B	4.05 ± 0.01^{aA}	4.05 ± 0.01^{aA}	4.04 ± 0.01^{aA}	4.03 ± 0.01^{aA}	4.02 ± 0.02^{aA}
	B-1	4.06 ± 0.01^{aA}	4.05 ± 0.01^{aA}	4.06 ± 0.01^{aA}	4.03 ± 0.01^{aA}	4.03 ± 0.02^{aA}
	A-9-1	3.91 ± 0.02^{bA}	3.90 ± 0.02^{bA}	3.87 ± 0.01^{bA}	3.76 ± 0.01^{bB}	3.62 ± 0.04^{bC}
	A-9	3.88 ± 0.07^{bA}	3.84 ± 0.03^{bAB}	3.80 ± 0.09^{bB}	3.70 ± 0.08^{bC}	3.64 ± 0.01^{bC}
Total acidity (g/100 g)	B	0.28 ± 0.28^{aA}	0.29 ± 0.39^{aA}	0.29 ± 0.38^{aA}	0.29 ± 0.39^{aA}	0.20 ± 0.38^{aA}
	B-1	0.29 ± .0.32^{aA}	0.29 ± 0.18^{aA}	0.29 ± 0.25^{aA}	0.30 ± 0.38^{aA}	0.31 ± 0.30^{aA}
	A-9-1	0.31 ± 0.14^{aA}	0.36 ± 0.32^{aAB}	0.42 ± 0.28^{aB}	0.53 ± 0.38^{aC}	0.63 ± 0.25^{aD}
	A-9	0.45 ± 0.38^{bA}	0.50 ± 0.25^{bAB}	0.56 ± 0.38^{bBC}	0.59 ± 0.38^{bC}	0.66 ± 0.52^{bD}
Reducing sugar content (g/100 g)	B	12.00 ± 0.03^{aA}	11.93 ± 0.02^{aB}	11.93 ± 0.01^{aB}	12.00 ± 0.01^{aB}	12.00 ± 0.02^{aB}
	B-1	11.86 ± 0.02^{aA}	11.8 ± 0.01^{bA}	11.73 ± 0.01^{bB}	11.86 ± 0.05^{aB}	11.86 ± 0.02^{aB}
	A-9-1	11.25 ± 0.02^{bA}	11.13 ± 0.01^{cB}	11.07 ± 0.02^{cBC}	11.02 ± 0.02^{bC}	10.43 ± 0.15^{bC}
	A-9	9.51 ± 0.13^{cA}	9.27 ± 0.14^{dB}	9.15 ± 0.11^{dC}	8.88 ± 0.05^{cD}	8.47 ± 0.18^{cD}
Total sugar content (g/100 g)	B	13.71 ± 0.10^{aA}	13.6 ± 0.10^{aA}	13.64 ± 0.15^{aA}	13.86 ± 0.07^{aB}	14.16 ± 0.10^{aC}

*Each value in the table is the mean ± standard deviation (SD) of three trials. Different lower and upper letters in each column and row, respectively indicate a statistically significant difference ($P < 0.05$).

** B: control sample, 1: 1000 µg/L of spiked diazinon, A: *L. acidophilus*, 9: Primary probiotic population (9 log CFU/mL).

Parameter	Treatment**	Refrigerated storage times (day)				
		0	7	14	21	28
	B-1	13.82 ± 0.05^{aA}	13.71 ± 0.10^{aABC}	13.64 ± 0.05^{aB}	13.80 ± 0.15^{aC}	14.16 ± 0.10^{aD}
	A-9-1	12.70 ± 0.03^{bA}	12.65 ± 0.02^{bB}	12.63 ± 0.05^{aB}	12.55 ± 0.02^{bBC}	12.50 ± 0.02^{bC}
	A-9	11.42 ± 0.05^{cA}	11.36 ± 0.11^{cAB}	11.33 ± 0.02^{bB}	11.30 ± 0.01^{cB}	11.14 ± 0.25^{cC}
Viability of probiotic (CFU/mL)	B	-	-	-	-	-
	B-1	-	-	-	-	-
	A-9-1	10.23 ± 0.04^{aA}	9.35 ± 0.01^{aB}	9.25 ± 0.02^{aC}	8.27 ± 0.01^{aD}	7.13 ± 0.02^{aE}
	A-9	10.85 ± 0.02^{bA}	9.96 ± 0.06^{bB}	9.67 ± 0.05^{bC}	8.92 ± 0.02^{bD}	7.88 ± 0.06^{bE}
Diazinon reduction (%)	B	-	-	-	-	-
	B-1	19.66^{aA}	38.40^{aB}	44.96^{aC}	49.86^{aD}	58.73^{aE}
	A-9-1	64.97^{bA}	49.60^{bB}	47.64^{bC}	45.04^{bD}	41.27^{bE}
	A-9	-	-	-	-	-

*Each value in the table is the mean ± standard deviation (SD) of three trials. Different lower and upper letters in each column and row, respectively indicate a statistically significant difference ($P < 0.05$).

** B: control sample, 1: 1000 µg/L of spiked diazinon, A: *L. acidophilus*, 9: Primary probiotic population (9 log CFU/mL).

As depicted in Table 6 the pH of probiotic apple juice samples (A-9-1 and A-9) decreased significantly during storage period from 3.91 and 3.88 to 3.62 and 3.64, respectively. However, the total acidity of A-9-1 and A-9 treatments increased significantly from 0.31 and 0.45 to 0.63 and 0.66 g mallic acid/100 g apple juice, respectively which is in good agreement with previous works (Ding & Shah, 2008; Pimentel et al., 2015). The reducing and total sugar concentration of probiotic apple juice samples (A-9 and A-9-1) showed a slight significant decline ($p < 0.05$) indicating sugar consumption for their growth and activity. Fortunately, the probiotic viability of apple juice samples decreased around 3 logarithmic cycle probably due to low temperature inappropriate for growth of *L. acidophilus* as well as post acidification of this strain. It is well-known fruit and vegetable juices are proper matrixes for delivery of probiotics because of their level of sugar, vitamins, antioxidant and minerals (Zoghi et al., 2017). Our results showed that, the probiotic viability of A-9 and A-9-1 samples decreased from 10.85 and 10.23 to 7.88 and 7.13 log CFU/mL, respectively. When it comes to diazinon concentration, this value for control sample (B-1) and

probiotic samples (A-9-1) declined from 803 to 412 µg/L and 153 to 0 µg/L, respectively at the end of storage time. It is worthy to note that 64.97, 49.60, 47.64, 45.04 and 41.27 % of diazinon reduction was related to probiotic effect via degradation or binding mechanism at day 0, 7, 14, 21 and 28 of cold storage, respectively. Results of this section are in good accordance with Zhoghi et al. (2017) study who investigated the effects of 6 weeks cold storage on patulin reduction by probiotic bacteria (Zoghi et al., 2017).

Conclusion

This study's results demonstrated that probiotic bacteria can act as diazinon reduction strategy in apple juice during fermentation and refrigerated storage. Diazinon-reducing ability of probiotic bacteria was species-dependent and *L. acidophilus* had the highest ability of diazinon removal. It was also observed that with increasing spiked diazinon concentration, the efficiency of probiotics in reducing diazinon decreased. Furthermore, non-viable (heat-killed) *L. acidophilus* cells also had the ability to reduce diazinon. After 72 hours of fermentation at 37 °C, the viability of *L. acidophilus* cells increased and level of diazinon in apple juice samples decreased significantly. Furthermore, during 28 days of cold storage at 4 °C, the amount of diazinon in treatments containing *L. acidophilus* decreased to zero. Also, the number of *L. acidophilus* cells decreased although it was still higher than the number required for being a healthy probiotic apple juice.

Declarations

Ethical Approval and Consent to Participate

Not applicable

Consent to Publish

Not applicable

Authors Contributions

All authors contributed to the study concept and design. Supervision, data curation and writing-review and editing: Amene Nematollahi; Formal analysis, data curation and writing original draft: Farahnaz rezaei; Methodology and project administration: Roghayeh Nejati and Mehran Sayadi. All authors read and approved the final manuscript.

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Competing Interests

No researchers have competing interest in this study.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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