

Investigating the antibacterial effects of some *Lactobacillus*, *Bifidobacterium* and *acetobacter* strains killed by different methods on *Streptococcus mutans* and *Escherichia coli*

Mohammad Sadegh Safari¹, Mehrnaz Keyhanfar^{1,*}, Rasoul Shafiei²

- 1) Department of Biotechnology, Faculty of Advanced Sciences and Technologies, University of Isfahan, Isfahan, Iran
2) Department of Biology, Faculty of Sciences, University of Isfahan, Isfahan, Iran

ABSTRACT

Although there are many health advantages assigned to different live bacteria such as probiotics, some health threatening effects have also been reported. For example, live bacteria can transfer antibiotic resistance genes to other commensal and opportunistic bacteria of gastrointestinal tract. Recently, it was shown that using killed bacteria have some advantages over live ones. In this research, heat, paraformaldehyde and ozone killing methods were used to kill the bacteria. *Acetobacter cerevisiae*, *Lactobacillus acidophilus*, *Bifidobacterium lactis* and traditional vinegar and fermented dairy product (Kumeh) derived bacteria were killed and their antibacterial activity against *Streptococcus mutans* and *Escherichia coli* was investigated. To identify the bacteria isolated from the traditional products, *16S rDNA* gene was partially sequenced. The gene analysis showed vinegar and Kumeh derived bacteria were *Acetobacter pasteurianus* and *Lactobacillus crustorum* (LcK) strains respectively. The *S. mutans* growth inhibition was detected in the all concentrations of all killed samples. However, generally, *E. coli* showed more resistant to the killed bacteria than *S. mutans* and the antibacterial effect of heat-killed bacteria against *E. coli* was not observed in the all concentrations for some killed bacteria. Among the pathogenic bacteria, *S. mutans* was the most sensitive one to the killed bacteria with 70% of reduction in its viability. In conclusion, this research showed that different killed bacteria had different effects on other bacteria and the killing method showed an impact on these effects. Overall, paraformaldehyde-killed *L. crustorum* (LcK) showed the best antibacterial activity against *S. mutans*; about 70% decrease in bacterial viability.

Keywords: *Acetobacter*; *Lactobacillus*; *Bifidobacterium*; killed bacteria

INTRODUCTION

Probiotics are live microorganisms, which when consumed in adequate amounts, confer a health effect on the host [1]. Elie Metchnikoff, a Russian scientist, suggested that lactobacilli might act against the putrefactive effects of gastrointestinal metabolism that contributed to diseases and aging [2]. *Lactobacillus* genus which was studied by Metchnikoff is categorized as probiotics now. Probiotics can confer health effect by adjusting gastrointestinal immune system, implicating such mechanism as production of antibacterial substances like bacteriocins, lactic

*Corresponding Author: Department of Biotechnology, Faculty of Advanced Sciences and Technologies, University of Isfahan, 81746-73441, Isfahan, Iran
Tel: +98-313-7934402 ; Fax: +98-313-7932342
E. mail: m.keyhanfar@ast.ui.ac.ir

acid and acetic acid [3,4]. They can also reduce colonization or decrease populations of pathogenic bacteria in the gastrointestinal tract and produce nutrients like vitamin K [5]. *Acetobacter* spp. are a subgroup of acetic acid bacteria which play an important role in food industry specially in vinegar production [6]. Recent studies have shown that these bacteria have some characteristic of probiotics and they are considered as potential probiotics [7].

According to the definition of “probiotic” being “alive” is crucial for a microorganism to be considered as probiotic. Some characteristics of probiotics, such as production of antimicrobial substances, effective adherence and colonization in intestinal tract, are related to their viability. Recent researches have shown that killed bacteria can be used instead of live microorganisms due to the risk of live bacteria. The administration of killed bacteria will cause losing some aspect of probiotics but it has some benefits for both probiotic producers and consumers [8]. For example, during storage of fermented probiotic products, special storage conditions are required to control the population of probiotic [9]. Another important issue about administration of live bacteria is probability of antibiotic resistance gene transfer among the gut microbiota [10]. Live microorganisms are able to transfer these genes by mechanisms like conjugation. Penicillin, vancomycin and tetracycline resistance have been identified in *lactobacillus* genus [11]. So, it seems important to avoid health risks by administration of the killed probiotics instead of live ones.

Different methods are available for killing bacteria. All cellular components can be affected by heat to some degree (outer layers, membrane, enzymes and proteins, DNA, RNA) [12]. Ozone is a powerful oxidizing agent which is known as GRAS (generally recognized as safe) [13]. Ozone has a destructive impact on polyunsaturated fatty acids of cell membrane and some amino acids like tyrosine, tryptophan, phenylalanine, and the sulphur containing amino acids like cysteine and oxidizes them [14,15]. Aldehydes such as paraformaldehyde are mostly used as fixative to stabilize the fine structural details of cells and tissues. The aldehyde group can react with nitrogen and some other atoms of proteins, forming cross link -CH₂- called a methylene bridge [16].

In this study, the antibacterial effect of heat killed, ozone killed, and paraformaldehyde killed *A. cerevisiae*, *L. acidophilus*, *B. lactis*, traditional vinegars and fermented dairy (Kumeh) derived bacteria were investigated against *Streptococcus mutans* and *Escherichia coli* by analyzing the growth of the bacteria following treatment with killed bacterial strains.

MATERIALS AND METHODS

Bacterial strains and growth conditions: In the current study, antibacterial effects of two diverse probiotic strains, *L. acidophilus* (ATCC 4356) and *B. animalis* subsp. *lactis* (DSM 10140) (both provided by the Department of Microbial Biotechnology, University of Isfahan, Iran), *A. cerevisiae* bacteria (LMG1625) (provided by Dr.R.Shafiei, from the Department of Microbiology, University of Isfahan, Iran.) and bacteria derived from traditional vinegar and Kumeh were tested. The probiotic strains were grown in de Man, Rogosa and Sharpe (MRS) agar at 37°C. For liquid culture, one colony was taken from MRS agar (a selective medium for lactobacillus strains) plate and grown 20 h in 50 ml of MRS broth at 37°C. The *A. cerevisiae* was grown in modified Carr selective medium (yeast extract 0.5%, peptone 1%, glucose 2%, absolute ethanol 3%, acetic acid 0.5%, ammonium phosphate 0.01%, magnesium sulfate 0.01%, and agar 1.5%) which is a specific isolation medium for *acetobacter* strains [17]. For liquid culture, one colony was taken from Carr agar plate and grown 24 h in 50 ml of Carr broth at 30°C. Three bacterial strains were isolated from Kumeh and two types of traditional vinegar (mulberry and grapes) using selective culture medium. The MRS broth medium was used to isolate bacteria from Kumeh and modified Carr broth medium to isolate bacteria from vinegars. The strains were cultivated on isolation media for 7 days followed by aseptically transferring 5 random colonies to solid medium to grow for 24 hours. The incubation temperature was 30°C

for all bacteria except for Kumeh isolated bacteria, incubated at 37°C. Morphological characteristics of selected colonies from each plate were checked out using Gram staining and catalase tests. Further experiments for identification and characterization by *16s rDNA* gene amplification of the isolated bacteria were carried out on just one of the colonies. Having prepared the growth rate curves of the bacteria (data are not shown), the bacteria were harvested in the exponential phase accordingly resuspended in PBS and centrifuged twice at 1800×g (Pars Azma Centrifuge, Iran) for 10 min. Bacterial concentration in PBS was obtained using a Thoma cell counting chamber and required concentrations (10^7 cell/ml, 10^6 cell/ml, 10^5 cell/ml) were prepared by diluting the main bacterial stocks.

Killing the bacteria: Heat killed bacteria were prepared by heating bacteria at 75°C for 15 min in a bain-marie. Each bacterial sample was exposed to ozone (by Opak ozone generator SIN_98_D) for 8 min. Paraformaldehyde 8% solution was prepared by dissolving 80 gr paraformaldehyde in 1000 ml PBS at 60°C Paraformaldehyde killed bacteria were prepared by incubating bacterial pellets with the paraformaldehyde solution with 1:1 ratio for 20 min at 4°C and then washed twice with PBS by centrifugation at 6000×g for 10 min. The samples of 0.1 mL of the killed bacteria were plated on MRS agar and Carr agar and incubated for 24 h at 37°C (at 30°C for *Acetobacter* strains) to confirm the effectiveness of the killing method.

DNA extraction: The bacterial cells were grown overnight (as mentioned above) and chromosomal DNA was isolated as described previously [18]. The aqueous phase contained purified DNA and was stored at -20°C and then was directly used for subsequent experiments.

PCR analysis of *16S rDNA*: PCR amplification of the partial *16S rDNA* gene of the isolated bacterial strains from Kumeh and two types of traditional vinegars were performed using the following universal forward and reverse primers: 27F: 5'AGA GTT TGA TCC TGG CTC AC3' [19] and 1492R: 5'CGG TTA CCT TGT TAC GAC TT3' [20]. The PCR mixture consisted of 25 µl of PCR master mix, 1 µl (10 pmole) of each primers, 3 µl of DNA template and 20 µl of DNase-RNase free water. The PCR reaction was performed in 50µl volumes under the following conditions: 94°C for 10 min, 35 cycles of (95°C for 1 min, 58°C for 45 s, 72°C for 1 min) and 72°C for 10 min. Amplification products were electrophoresed on 1% agarose gel and visualized using the safe stain under UV light [21]. The 1200 bp sequences were PCR amplified and sequenced by Gen Fanavaran Ltd. All the sequences were analyzed by CLC Main workbench 5 program and were searched for homologies in the BLASTN database (National Center for biotechnology Information) using the basix local alignment search tool.

Phylogenetic Analysis: After sequencing the amplicons, to compare the similarities among the obtained sequences the phylogenetic trees were constructed by the neighbor-joining statistical method using Mega version 6. The sequence data were sampled 1,000 times for bootstrap analysis with 50% cut-off. *Gluconobacter oxydans* strain DSM 3503 and *Bacillus subtilis* subsp. *spizizenii* strain NBRC 101239, were used as outgroups.

Antibacterial activity assay: Screening of all killed bacteria for their qualitative antibacterial activity was performed by employing microdilution method [22]. One ml cultures of *S. mutans* (ATCC 35668) and non-pathogenic *E. coli* (ATCC 25922) (both obtained from the Department of Microbial Biotechnology, University of Isfahan, Iran) were prepared and incubated overnight at 37°C. The overnight cultures were diluted by Muller-Hinton broth [23] to yield a density of bacterial cells about 1.5×10^7 cells/ml (0.05 McFarland). 50 µl of the bacterial suspension was added to each well of a 96 wells plate. Then, 100µl of cation adjusted Muller Hinton Broth and 100 µl of each killed bacterial samples with ratio of 2:5 were added to each well. One well was kept as positive control, contained broth, inoculum and tetracycline (1 mg/ml), and one well as negative control, contained broth and inoculum and one well (just

broth) to make sure there is no contamination in the broth. The plates were covered and incubated overnight at 37°C. To indicate the bacterial growth inhibition, optical density of the wells were measured by multiwell scanning spectrophotometer (ELISA reader) at 580nm.

Lactic acid analysis by Gas Chromatography: Supernatant of Lck bacteria was analyzed to assure that bacterial sample does not contain lactic acid. The paraformaldehyde-killed LcK sample soluble was centrifuged at 8000×g for 2 min. The supernatant was collected and then filtrated by 0.22 µm pore size membrane filter. One µl of the filtrate was injected to GC (Aglinet, model 6890N) equipped with INNOWAX column and the concentration of lactic acid was assessed.

Statistical Analyze: The results for inhibition tests were expressed as the mean±SD of three independent experiments. The data were analyzed by one-way ANOVA test with IBM SPSS Statistics. A P<0.05 was considered to represent a statistically significant difference between compared data sets.

RESULTS

The isolated bacterium from Kumeh was rod-shaped, Gram-positive, and catalase negative with circular, smooth, and white colonies on MRS agar. In figure 1, S1 shows PCR of amplicon for Kumeh derived bacteria (molecular size of 1200 bp) was associated with the calculated size of a target gene and shows the single band of PCR. Table 1 (in supplement file) presents the DNA sequences target genes presents. Partial sequencing of *16S rDNA* indicated 99% identity with *L. crustorum*. Therefore the isolated bacterium was confirmed as *L. crustorum* and abbreviated as LcK in this study. The phylogenetic placement of strain isolated from Komeh is shown in Figure 2.

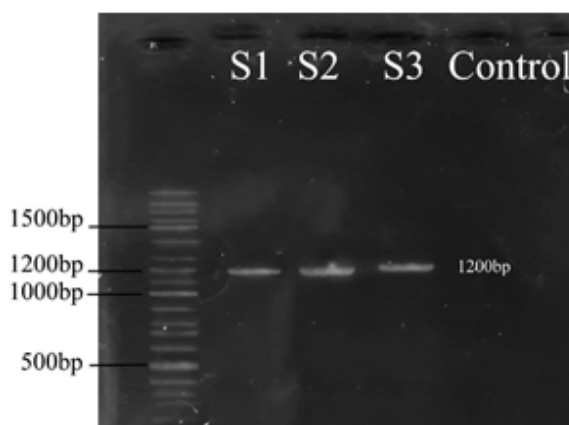


Figure 1: Gel electrophoresis of PCR for Lck (S1), ApG (S2), and ApM (S3)

The isolated bacteria from mulberry and grapes vinegars were Gram negative coccobacilli and catalase positive with circular, rough, and white colonies on MRS agar. In figure 1, S2 and S3 show PCR of amplicon for mulberry and grapes vinegars derived bacteria (molecular size of 1200 bp) was associated with the calculated size of a target gene and shows the single band of PCR. Partial sequencing of *16S rDNA* of both isolated bacteria indicated 99% identity with *Acetobacter pasteurianus* species. Therefore the isolated bacterium were confirmed as strains of *A. pasteurianus* and abbreviated as ApM (Mulberry vinegar isolated strain) and ApG (Grape vinegar isolated strain) in this paper. The phylogenetic placement of strains isolated from mulberry and grape vinegar is shown in Figure 3.

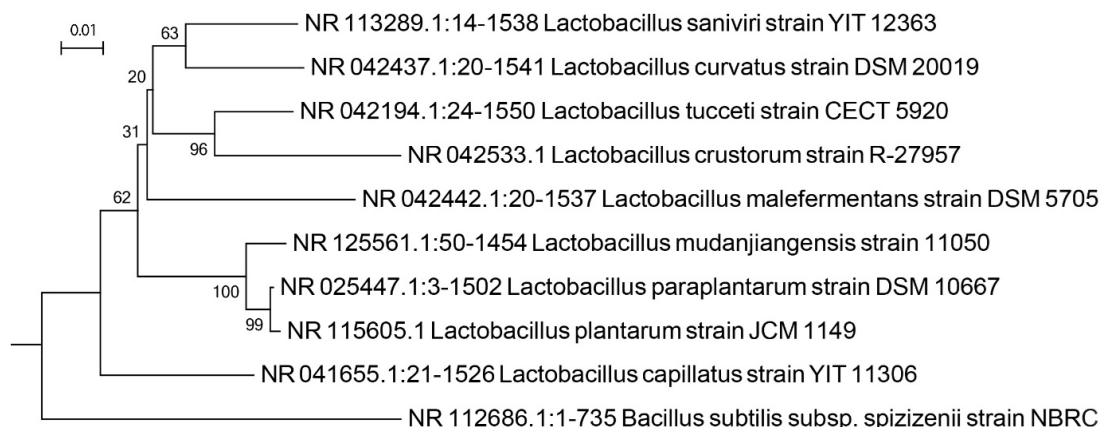


Figure 2: Phylogenetic tree based on *16S rDNA* sequence analysis, showing the phylogenetic placement of strain isolated from komeh (LcK). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree was rooted on *Bacillus subtilis*.

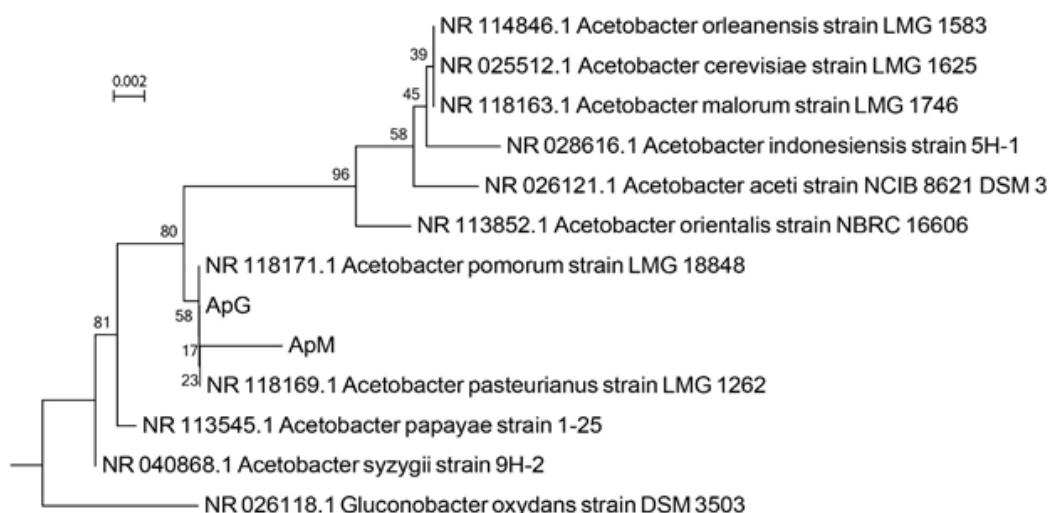


Figure 3: Phylogenetic tree based on *16S rDNA* sequence analysis, showing the phylogenetic placement of strains isolated from mulberry (ApM) and grape vinegar (ApG). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. the tree was rooted on *Gluconobacter oxydans*.

Antibacterial activity of the killed bacteria were examined using two different bacterial pathogens, including a Gram-positive strain, *S. mutans* and a Gram-negative bacteria strain, *E. coli*. The growth inhibition was verified after 24 h of incubation and as shown in Figure 4a, all of the heat-killed bacteria had antibacterial and growth inhibition activity but among them, Lck expressed the best results on *S. mutans*; about 65% ($P < 0.05$) decrease in bacterial growth in each examined concentrations. As it is shown in Figure 4a, there was no significant difference between concentrations' growth inhibition activity in each killed sample and the effect was not dependent to the concentration of killed bacteria (Ozone-killed, Heat-killed, and Paraformaldehyde-killed). Three samples of ozone killed bacteria, *A. cerevisiae*, *L. acidophilus* and Lck, had almost similar effect on *S. mutans* (Fig. 4b). A 60% decrease in bacterial growth was seen as a result of applying these samples. All the paraformaldehyde killed bacteria also revealed great significant impact on bacterial growth of *S. mutans*. Similar to heat killed bacteria, Lck expressed the best antibacterial effect and caused 70% decrease in bacterial growth (Fig. 4c).

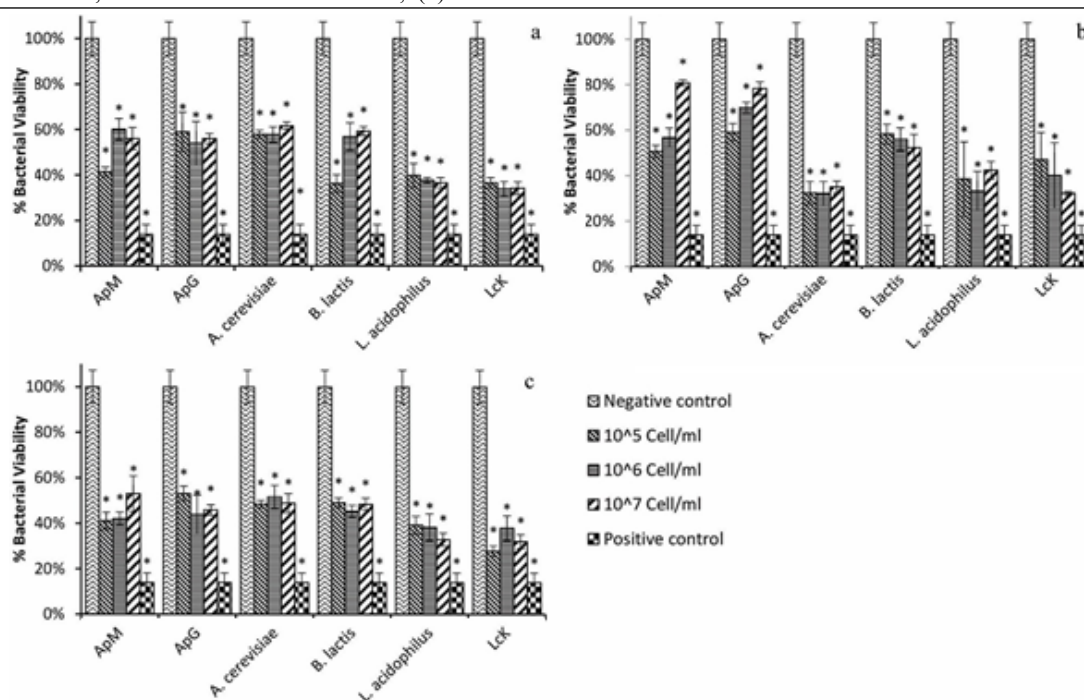


Figure 4: Antibacterial effect of different concentrations of a) heat-killed, b) Ozone killed, and c) paraformaldehyde killed bacteria on *S. mutans* ATCC35668. The *S. mutans* bacteria were grown in 96 well plates, and were cultured in absence (Negative control) or presence of killed bacteria. These results were obtained as described above, and the relative proportion % of untreated (negative control) bacteria at 24 h. Bars, means of triplicates \pm SD. * $P < 0.05$, as compared with the relative growth of control sample.

In contrast to the effect of killed bacteria on *S. mutans* which was able to be detected in all concentrations of all samples, antibacterial effect of heat-killed bacteria against *E. coli* was not observed in some situations. Generally, *E. coli* showed more resistant to the killed bacteria than *S. mutans*. The best antibacterial activity of the heat-killed bacteria was observed in *B. lactis* which decreased bacterial growth about 20% (Fig. 5a). Ozone killed bacteria showed similar effect on *E. coli* and again the best result was achieved by *B. lactis* with 30% decrease in bacterial growth (Fig. 5b). The paraformaldehyde killed bacteria had greater impact on *E. coli* viability in comparison with other methods. All the killed bacteria decreased *E. coli* growth about 40% (Fig. 5c).

DISCUSSION

Fermented foods such as dairy products and vinegar have been recognized as valuable products to human health. Many species and strains of lactic acid bacteria and acetic acid bacteria were isolated and identified from these products. Partial sequencing of *16S rDNA* gene of isolated bacteria confirmed that Kumeh isolated bacteria as *L. crustorum* and vinegar isolated bacteria as *A. pasteurianus*. Although these bacteria have shown some aspects of probiotics, they are not well known as probiotics and further studies are required [7].

The *S. mutans* is considered to play an important role in dental caries [24]. Probiotic containing products are known to contain compounds that reduce the risk of dental caries [25]. Antibacterial effects of different probiotics on *Streptococcus* species were investigated in other studies. For instance, *B. animalis* subsp. *Lactis* DN-173010 significantly reduced the salivary levels of *S. mutans* in orthodontic patients [26]. A statistically significant reduction of the *S. mutans* levels was observed after consumption of the bacteria via the straw and the tablets [27]. The effect of *Lactobacillus* GG on *S. sobrinus* and their experiments showed a 10-fold inhibition in growth of *S. sobrinus* by *Lactobacillus* GG substances [28]. *Lactobacillus* and

Bifidobacterium species had moderate antibacterial activity, a growth inhibition from 20% to 70% (Fig. 3). In addition, Lck expressed the best antibacterial effect and caused 70% decrease in *S. mutans* viability (Fig. 3c).

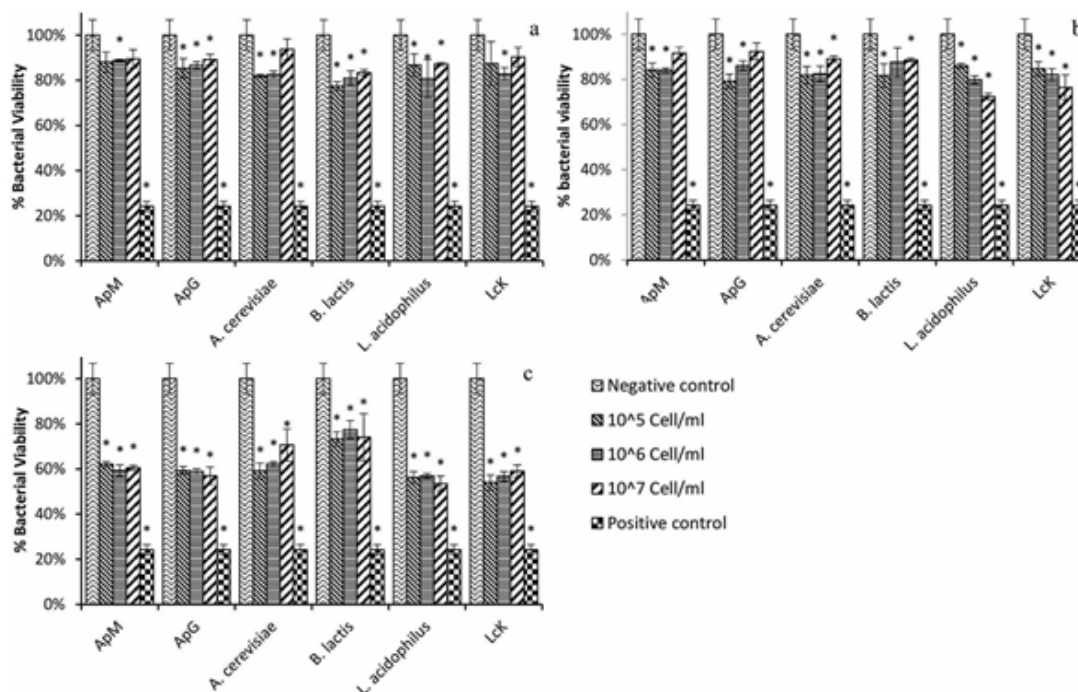


Figure 5: Antibacterial effect of different concentrations of **a)** heat-killed, **b)** ozone killed, and **c)** paraformaldehyde killed on *E. coli* ATCC25922. The *E. coli* bacteria were grown in 96 well plates, and were cultured in absence (Negative control) or presence of killed bacteria. These results were obtained as described above, and the relative proportion % of untreated (negative control) bacteria at 24 h. Bars, means of triplicates \pm SD. *P<0.05, as compared with the relative growth of control sample.

Recent researches described a new enzymatic functionality for the S-layer (surface layer) of *L. acidophilus* ATCC 4356. The S-layer showed an endopeptidase activity against the cell wall of *S. enterica* [29]. Moreover, S-layer acted synergistically in combination with nisin to inhibit the growth of *S. enterica*, *Staphylococcus aureus* and *Bacillus cereus*. [30]. The growth of isolated mutans streptococci strains was also inhibited by *Lactobacillus* strains at bacterial concentration of $\geq 10^7$ CFU/ml [28]. Schwendicke et al. assessed antibacterial activity of viable and heat-inactivated *B. animalis* BB12 *in vitro*. Viable *B. animalis* did not significantly reduce cariogenicity of *S. mutans*, whilst heat-inactivated *B. animalis* decreased cariogenicity of *S. mutans* in dentinal cavities [31]. Our data imply that all of the killed bacteria with different methods expressed antibacterial activity against *S. mutans*. However, a better antibacterial activity was shown when bacteria were killed with paraformaldehyde in comparison with heat killed and ozone killed bacteria.

Coconnier et al investigated the antibacterial effect of the adhering human *L. acidophilus* strain LB against a wide range of gram-negative and gram-positive pathogens and showed *L. acidophilus* decreased the viability of *E. coli*, *S. typhimurium*, *S. flexneri*, *B. cereus*, *P. aeruginosa*, and *Enterobacter* spp. without any significant inhibition of other lactobacilli and bifidobacteria [32]. Inoculation of yoghurt and soymilk sample containing viable *B. lactis* (Bb-12) and *B. longum* (Bb-46) with *E. coli* or *S. aureus* showed more than 90% decrease in the count of *E. coli* [33]. In the current study, most of the killed bacteria had antibacterial effect on *E. coli*. There is also significant difference between killing methods. Paraformaldehyde killed bacterial strains showed more antibacterial activity in comparison with other methods which was up to 40% loss of growth. It is probably because paraformaldehyde has less damage on bacterial proteins and other biological structures.

Viable probiotics may suppress the proliferation of pathogens by different methods. They can produce diverse antimicrobial metabolites including bacteriocins, lactic acid and acetic acid [34]. In this study, killed bacteria were used to assay their antibacterial activity. According to GC analysis, there was no lactic acid present in the inoculum, so the antibacterial effect cannot be admitted to these metabolites. To the best of our knowledge, there is no evidence that shows acetobacters can produce bacteriocins, so the antibacterial activity of killed bacteria is probably related to the killed bacterial extract structures such as lipopolysaccharide, inner and outer membrane proteins, peptidoglycan and etc. In conclusion, in this research it was shown that the studied killed bacteria had different effects on other bacteria -mostly growth inhibitory effect- and the method of killing had an impact on these effects. Although the mechanism(s) by which the selected probiotics and acetic acid bacteria reduced the viability of pathogenic bacteria remains to be elucidated, this study indicate that killed bacteria components could be an important factor which has some impacts on the homeostasis of the flora in the gastrointestinal tract. Further studies are needed to clarify the mechanism(s) of this effect.

Acknowledgements: The authors are thankful to Eghlidi Research & Engineering Group, for providing laboratory facilities (Ozone generator).

Conflict of Interest: The authors declare that they have no conflict of interest.

REFERENCES

1. Reid G. The importance of guidelines in the development and application of probiotics. *Curr Pharm Des* 2005;11:11-16.
2. Gasbarrini G, Bonvicini F, Gramenzi A. Probiotics history. *J Clin Gastroenterol* 2016; 50:S116-S119.
3. Behnsen J, Deriu E, Sassone-Corsi M, Raffatellu M. Probiotics: properties, examples, and specific applications. *Cold Spring Harb Perspect Med* 2013;3:211-268.
4. Chapman CM, Gibson GR, Rowland I. Health benefits of probiotics: are mixtures more effective than single strains? *Eur J Nutr* 2011;50:1-17.
5. Hardy H, Harris J, Lyon E, Beal J, Foey AD. Probiotics, prebiotics and immunomodulation of gut mucosal defences: homeostasis and immunopathology. *Nutrients* 2013; 5:1869-1912.
6. Sengun IY, Karabiyikli S. Importance of acetic acid bacteria in food industry. *Food Control* 2011;22:647-656.
7. Haghshenas B, Nami Y, Abdullah N, Radiah D, Rosli R, Khosroushahi AY. Anticancer impacts of potentially probiotic acetic acid bacteria isolated from traditional dairy microbiota. *LWT- Food Sci Technol* 2015;60:690-697.
8. Lahtinen SJ. Probiotic viability-does it matter? *Microb Ecol Health Dis* 2012;23:18567.
9. Jakobsen M, Narvhus J. Yeasts and their possible beneficial and negative effects on the quality of dairy products. *Int Dairy J* 1996;6:755-768.
10. Sharma P, Tomar SK, Goswami P, Sangwan V, Singh R. Antibiotic resistance among commercially available probiotics. *Food Res Int* 2014;57:176-195.
11. Tynkkynen S, Singh KV, Varmanen P. Vancomycin resistance factor of *Lactobacillus rhamnosus* GG in relation to enterococcal vancomycin resistance (van) genes. *Int J Food Microbiol* 1998;41:195-204.
12. Russell AD. Lethal effects of heat on bacterial physiology and structure. *Sci Prog* 2003; 86:115-137.
13. Isikber AA, Athanassiou CG. The use of ozone gas for the control of insects and microorganisms in stored products. *J Stored Prod Res* 2015;64:139-145.
14. Uzun H, Ibanoglu E, Catal H, Ibanoglu S. Effects of ozone on functional properties of proteins. *Food Chem* 2012;134:647-654.

15. Tiwari B, Brennan CS, Curran T, Gallagher E, Cullen P, O'Donnell C. Application of ozone in grain processing. *J Cereal Sci* 2010;51:248-255.
16. Kiernan JA. Formaldehyde, formalin, paraformaldehyde and glutaraldehyde: what they are and what they do. *Micros Today* 2000;8:8-13.
17. Maal KB, Shafiei R, Kabiri N. Production of apricot vinegar using an isolated *Acetobacter* strain from Iranian apricot. *World Acad Sci Eng Technol* 2010;4:162-4.
18. Cheng HR, Jiang N. Extremely rapid extraction of DNA from bacteria and yeasts. *Biotechnol Lett* 2006;28:55-59.
19. Marchesi JR, Sato T, Weightman AJ, Martin TA, Fry JC, Hiom SJ, Wade WG. Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. *Appl Environ Microbiol* 1998;64:795-799.
20. Turner S, Pryer KM, Miao VP, Palmer JD. Investigating deep phylogenetic relationships among cyanobacteria and plastids by small subunit rRNA sequence analysis. *J Eukaryotic Microbiol* 1999;46:327-338.
21. Dehghanifar S, Keyhanfar M, Emtiazi G. Production and partial purification of thermostable bacteriocins from *Bacillus pumilus* ZED17 and DFAR8 strains with antifungal activity. *Mol Biol Res Commun* 2019;8:41-49.
22. Wikler MA. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically: approved standard. CLSI (NCCLS) 2006;26:M7-A7.
23. Fani MM, Kohanteb J, Dayaghi M. Inhibitory activity of garlic (*Allium sativum*) extract on multidrug-resistant *Streptococcus mutans*. *J Indian Soc Pedod Prev Dent* 2007;25:164-168.
24. Hamada S, Slade HD. Biology, immunology, and cariogenicity of *Streptococcus mutans*. *Microbiol Rev* 1980;44:331-384.
25. Ahola AJ, Yli-Knuutila H, Suomalainen T, Poussa T, Ahlström A, Meurman JH, Korpela R. Short-term consumption of probiotic-containing cheese and its effect on dental caries risk factors. *Arch Oral Biol* 2002;47:799-804.
26. Cildir SK, Germec D, Sandalli N, Ozdemir FI, Arun T, Twetman S, Caglar E. Reduction of salivary mutans streptococci in orthodontic patients during daily consumption of yoghurt containing probiotic bacteria. *Eur J Orthod* 2009;31:407-411.
27. Caglar E, Cildir SK, Ergeneli S, Sandalli N, Twetman S. Salivary mutans streptococci and lactobacilli levels after ingestion of the probiotic bacterium *Lactobacillus reuteri* ATCC 55730 by straws or tablets. *Acta Odontol Scand* 2006;64:314-318.
28. Meurman JH, Antila H, Korhonen A, Salminen S. Effect of *Lactobacillus rhamnosus* strain GG (ATCC 53103) on the growth of *Streptococcus sobrinus* in vitro. *Eur J Oral Sci* 1995; 103:253-258.
29. Prado Acosta M, Mercedes Palomino M, Allievi MC, Sanchez Rivas C, Ruzal SM. Murein hydrolase activity in the surface layer of *Lactobacillus acidophilus* ATCC 4356. *Appl Environ Microbiol* 2008;74:7824-7827.
30. Prado-Acosta M, Ruzal SM, Allievi MC, Palomino MM, Sanchez Rivas C. Synergistic effects of the *Lactobacillus acidophilus* surface layer and nisin on bacterial growth. *Appl Environ Microbiol* 2010;76:974-977.
31. Schwendicke F, Horb K, Kneist S, Dörfer C, Paris S. Effects of heat-inactivated *Bifidobacterium* BB12 on cariogenicity of *Streptococcus mutans* in vitro. *Arch Oral Biol* 2014;59:1384-1390.
32. Coconnier MH, Liévin V, Bernet-Camard MF, Hudault S, Servin AL. Antibacterial effect of the adhering human *Lactobacillus acidophilus* strain LB. *Antimicrob Agents Chemother* 1997;41:1046-1052.
33. Abd El-Gawal IA, El-Sayed EM, El-Zeini HM, Hafez SA, Saleh FA. Antibacterial Activity of Probiotic Yoghurt and Soy-Yoghurt against *Escherichia coli* and *Staphylococcus aureus*. *J Nutr Food Sci* 2014;4:1-6.
34. Spinler JK, Taweechotipatr M, Rognerud CL, Ou CN, Tumwasorn S, Versalovic J. Human-derived probiotic *Lactobacillus reuteri* demonstrate antimicrobial activities targeting diverse enteric bacterial pathogens. *Anaerobe* 2008;14:166-171.