Effects of Penicillin G on Morphology and Certain Physiological Parameters of Lactobacillus acidophilus ATCC 4356

Khaleghi, M.1*, R. Kasra Kermanshahi2, and S. H. Zarkesh-Esfahani3

1Department of Biology, Faculty of Sciences, Shahid Bahonar University of Kerman, Kerman, Iran
2Department of Biology, Faculty of Sciences, Alzahra University, Tehran, Iran
3Department of Biology, Faculty of Sciences, University of Isfahan, Isfahan, Iran

Evidence shows that probiotic bacteria can undergo substantial structural and morphological changes in response to environmental stresses, including antibiotics. Therefore, this study investigated the effects of penicillin G (0.015, 0.03, and 0.06 mg/l) on the morphology and adhesion of Lactobacillus acidophilus ATCC 4356, including the colony morphotype, biofilm production, hydrophobicity, H2O2 formation, S-layer structure, and slpA gene expression. Whereas only smooth colonies grew in the presence of penicillin, rough and smooth colony types were observed in the control group. L. acidophilus ATCC 4356 was found to be hydrophobic under normal conditions, yet its hydrophobicity decreased in the presence of the antibiotic. No biofilm was produced by the bacterium, despite testing a variety of different culture conditions; however, treatment with penicillin G (0.015–0.06 mg/l) significantly decreased its production of H2O2, formation and altered the S-layer protein structure and slpA gene expression. The S-protein expression decreased with 0.015 mg/l penicillin G, yet increased with 0.03 and 0.06 mg/l penicillin G. In addition, the slpA gene expression decreased in the presence of 0.015 mg/l of the antibiotic. In conclusion, penicillin G was able to alter the S-layer protein production, slpA gene expression, and certain physicochemical properties of Lactobacillus acidophilus ATCC 4356.

Key words: Lactobacillus acidophilus, S-layer, slpA gene, morphology, hydrophobicity, penicillin

Lactic acid bacteria (LAB) species, including lactobacilli and bifidobacteria, have a “generally regarded as safe” (GRAS) status and are frequently used as probiotics [14, 15, 36, 39, 43]. Adhesion to intestinal epithelial cells is an important prerequisite for the colonization of probiotic strains in the gastrointestinal tract [1, 30]. External structures of bacterial cells, such as S-layer, fibronectin, and mucin-binding proteins, may play a role in bacterial cell adhesion [1, 2, 20, 30, 42, 47]. In this regard, some functions have already been reported or assumed for S-layer proteins [7, 23, 42]. Many species of the genus Lactobacillus possess an S-layer [2], and these proteins can mediate attachment to intestinal epithelial cells [2, 20, 30]. There is also increasing evidence that S-layer-carrying bacteria may use S-layer variation, by expressing alternative S-layer protein genes, for adaptation to different stress factors, such as the immune response of the host to pathogens and drastic changes in the environmental conditions for nonpathogens [10, 20, 23, 40]. The presence of multiple S-layer protein genes seems to be quite common for lactobacilli, such as Lactobacillus acidophilus ATCC 4356, Lactobacillus brevis ATCC 14869, and Lactobacillus crispatus ICM 5810 [2, 5, 23]. Boot et al. [6, 8] identified two S-layer protein-encoding genes, slpA and slpB, where slpA is active and slpB is silent under normal growth conditions. The slpA gene is interchanged with the slpB gene through inversion of a chromosomal fragment in a fraction of a culture (0.3% of cell growth under laboratory conditions) [8, 10].

Lactobacilli can also produce H2O2, which can inhibit or kill other microbes and pathogens, particularly those that lack or have low levels of H2O2-scavenging enzymes [17]. To dominate the vaginal ecosystem, recent data suggest that H2O2 production by lactobacilli may be more important than lactic acid production [37].

Microorganisms must often cope with hostile environmental conditions [5, 48]. Phenotypically, lactobacilli respond to altered growth conditions by morphological changes that become apparent microscopically or colonially on solid media [1, 29]. In addition, the surface properties of microorganisms are dependent on the medium and growth conditions [1, 5,
Materials and Methods

Bacterial Strain, Medium, Growth, and Storage

*L. acidophilus* ATCC 4356 was obtained from the German Type Culture Collection, cultivated in an MRS broth (Scharlau, Spain), and stored as described previously [7, 19, 20].

Determination of Minimum Inhibitory Concentration (MIC)

The MIC of penicillin G was determined using bacterial broth dilution methods according to the method used by Baron and Finegold [4].

Penicillin-Treated Medium

To study the effect of penicillin G on an MRS broth and MRS agar (Merck, Germany) (pH=6.5) containing 0.015, 0.03, and 0.06 mg/l penicillin G (Jaberebe Hayan Co., Iran) were prepared. MRS broth and agar without penicillin G were used as the control media. To compare the bacterial growth in the presence of penicillin G and in the control, the bacteria were cultured under anaerobic conditions in a jar using an Anaerocult A-strip (Merck, Germany) at 37°C for 18 h, and then inoculated into fresh media [1% (v/v)]. Thereafter, the bacterial cultures were counted as described previously [20].

Determination of Colony Morphotype

The colony morphology of *L. acidophilus* ATCC 4356 was determined using stereomicroscopy (ZEISS/ Stemi SVII) after culturing the media and growth conditions on the physicochemical surface properties of several *Lactobacillus* species [12, 21, 29, 30, 33, 41], *Bifidobacterium bifidum* [21], and *Pseudomonas aeruginosa* [28].

Probiotics have to be systematically examined for antibiotic susceptibility in order to avoid the spread of antibiotic-resistant determinants by the food chain. Various reporters have already been established for determining the susceptibility of some *Lactobacillus* and *Bifidobacterium* species to certain antimicrobial agents. For example, *Lactobacillus acidophilus* has been shown to be sensitive to many antibiotics, such as penicillin G [15, 18, 24].

Therefore, the present study investigated the effects of the antibiotic penicillin G on the morphotype, colony formation, S-layer production, expression of the *slpA* gene, and certain other physicochemical characteristics of *Lactobacillus acidophilus* ATCC 4356.

Bacterial Hydrophobicity Through Interfacial Adhesion

The MATH (microbial adhesion to hydrocarbons) test was carried out as described previously [30, 35, 41], using xylene as the hydrophobic solvent. The percentage of bacterial adhesion to the solvent was calculated using the following formula:

\[
\% \text{ hydrophobicity} = \left( \frac{A_0 - A_2}{A_0} \right) \times 100
\]

where

- \( A_0 \) = absorbance before adding solvent at 600 nm.
- \( A_2 \) = absorbance of aqueous phase after adding solvent at 600 nm.

The MATS (microbial adhesion to solvents) values were obtained using two different solvents, chloroform (Merck, Germany) and ethyl acetate (Merck, Germany) [30, 35].

Measurement of H₂O₂ Production

The H₂O₂ concentrations were measured as described previously [34].

Isolation of S-layer Protein

To isolate the S-layer, extract the total RNA, and assess the *slpA* gene expression, the cells were harvested while they were growing exponentially (OD₆₀₀ ≈ 0.4). The S-layer protein was isolated according to Boot et al. [7], and *L. casei* ATCC 393 used as the negative control.

SDS-PAGE Analysis

The SDS-PAGE of the protein samples was carried out according to Sambrook and Russell [38], using the Precision Plus Protein Standard [low molecular weight marker (10−250 kDa), Biorad, UK] as the molecular marker. The samples were run on a 12% polyacrylamide gel at 100 V, and the protein bands visualized by Coomassie blue staining.

Measurement of Total Protein

After isolating the S-protein using 4.0 M guanidine hydrochloride, the supernatant was dialyzed against distilled water at 4°C and its protein concentration determined according to Bradford’s method [11].

Isolation of Total RNA

The *L. acidophilus* ATCC 4356 cells were grown in an MRS broth until they reached an optical density of around OD₆₀₀=0.4. The cells were then harvested by centrifugation (5,000 × g for 10 min at 4°C) [8] and washed with ice-cold TE buffer. The total RNA was isolated using a protective RNeasy Minikit (Qiagen) according to the manufacturer’s recommendations, and then treated with Deoxyribonuclease I (DNase I, RNase-free; Fermentas) at 37°C for 30 min according to the manufacturer’s recommendations.

RT-PCR

The reverse transcription (RT) of the RNA samples was performed with 150 ng of total RNA and 0.5 µg of oligo dT primer using a First Strand cDNA Synthesis kit (Fermentas) at 42°C for 60 min, as recommended by the manufacturer.

Forward and reverse primers were designed for the *slpA* gene of *L. acidophilus* ATCC 4356 as follows: *slpA* forward (5'-TGG CCG TTC TTT AAT GTG TA-3') and *slpA* reverse (5'-ACA TCA ACG CTG CAA ACA TC-3'). These primers generated a 154 bp PCR product in the PCR reaction.
16S rRNA was used as the internal control gene based on previously reported primers [44] that generate a 370 bp PCR product.

The final volume of the PCR reaction was 25 µl, with the following components: 1 µl cDNA (≈ 7.5 ng), 1 µl (100 pmol/µl) from each primer, 0.5 µl dNTPs mix, 0.5 µl MgCl₂, and 0.25 µl (5 U/µl) Taq DNA polymerase (Fermentas).

The Mastercycler (Eppendorf) was programmed as follows: initial denaturation for 5 min at 94°C; 30 cycles at 94°C for 45 s, 54°C for 30 s, 72°C for 30 s; and a final extension at 72°C for 8 min. The PCR products (and 50 bp DNA ladder; Fermentas) were separated on a 1% agarose gel and visualized by ethidium bromide staining.

**Statistical Assessment**

All the experiments and measurements were repeated at least three times. All the statistical analyses were performed using SPSS and Excel 2003 softwares. All the experimental results were analyzed using mean descriptive statistics, the correlation coefficient, and a single-factorial analysis of variance. A value of P<0.05 was regarded as statistically significant.

**RESULTS AND DISCUSSION**

**Assessment of MIC**

*Lactobacillus acidophilus* ATCC 4356 was found to be susceptible to penicillin G with an MIC of 0.12 mg/l (data not shown). Therefore, three concentrations of penicillin G (0.015, 0.03, and 0.06 mg/l) were chosen for further experiments.

**Effect of Penicillin G on Colony Morphotypes of *L. acidophilus***

After incubating *L. acidophilus* ATCC 4356 in an MRS agar (with or without penicillin G) for 72 h under anaerobic conditions at 37°C, diverse colony types appeared on the surface of the MRS agar plates. The control plates (without penicillin G) revealed two colony morphotypes, R (Rough) and S (Smooth), with approximately 2-fold more R-type colonies than S-type colonies. The R-type colonies were large, irregular, and flat to umbonate with a matte surface and mottled opacity. In contrast, the S-type colonies were smaller, circular with a smooth edge, and convex with a glistening translucent appearance (Fig. 1A).

Gram staining showed that the R-type colonies were composed of a mixture of long and short Gram-positive rods in long chains and filaments in tangled masses of cells (Fig. 1B), whereas the S-type colonies were composed of a mixture of long and short Gram-positive rods in single cells (Fig. 1C).

For the MRS agar containing 0.015–0.06 mg/l penicillin G, only S-type colonies appeared, which were smaller than those in the controls. Evidently, the resistance of the R- and S-type colonies to stress conditions was different. As such, the S-type colonies were more stable in the presence of penicillin G, as the MRS agar containing penicillin G (0.015–0.06 mg/l) was dominated by S-type colonies.

This finding is also in agreement with previous studies by Altermann *et al.* [1], Bron *et al.* [12], Khaleghi *et al.* [26], and Klaenhammer and Kleeman [29].

The results of this section showed that when increasing the level of penicillin G, the number of colonies decreased and the bacterial morphology changed. In the presence of penicillin G, two kinds of cell morphology were observed: thin and faint-colored cells, and thick and spiral cells (Fig. 2).

Microorganisms must often cope with hostile environmental conditions, and to do so they have developed sophisticated...
cooperative behavior and intricate communication capabilities. In some cases, these capabilities are exploited by bacterial colonies to develop complex patterns in response to adverse growth conditions [5]. Evidence already exists that phenotypic variability is caused by various conditions (e.g., substrate composition, culture history, and conditions of growth) [1, 5, 12, 16, 24, 26, 29]. Varmer et al. [46] found that PBPs (penicillin-binding proteins) are required for the formation of spiral cells. However, the affinities of PBPs to penicillin G may be different, and some PBPs may decrease, increase, or even vanish under different conditions [13]. Different receptors have different affinities to a drug and each can mediate a different effect. For example, the attachment of penicillin to one PBP may result in abnormal elongation of the cell, whereas attachment to another PBP may lead to a defect in the periphery of the cell wall, resulting in cell lysis [25].

Biofilm Formation
Kirisits et al. [28] reported that different types of environmental pressure determine the colony morphology variants of P. aeruginosa, and many of these variants also have biofilm-related phenotypes. Therefore, this study investigated the biofilm formation by L. acidophilus ATCC 4356 using adhesion to glass slides and microtiter plate techniques. The results indicated no biofilm production by L. acidophilus ATCC 4356 on either the glass slides or the microtiter plates after 72 h. In contrast, Staphylococcus aureus PTCC 1431, the control bacteria, did produce a biofilm, as detected by both methods (data not shown). Despite testing a wide range of different conditions, L. acidophilus ATCC 4356 did not produce a biofilm. Thus, it was concluded that the modification of the colony and cell morphology was not dependent on biofilm formation.

Hydrophobic Characteristics of L. acidophilus ATCC 4356
The results of the MATH test using xylene indicated that L. acidophilus ATCC 4356 is a hydrophobic bacterium. The hydrophobicity of the bacteria decreased in the presence of penicillin G and reached the lowest level in the presence of a high concentration (0.06 mg/l) of penicillin G (Fig. 3). The bacterial hydrophobicity was the highest in the control group and the lowest in 0.06 mg/l penicillin G (Table 1). Variations in the surface properties of L. acidophilus have already been reported [2, 30, 35, 41]. To define hydrophobic characteristics, bacteria are divided into 3 categories: bacteria with high (71–100%), medium (36–70%), and low (0–35%) hydrophobicities [35]. In this study, the bacterial

<table>
<thead>
<tr>
<th>Media</th>
<th>Xylene</th>
<th>Chloroform</th>
<th>Ethyl acetate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>85.98</td>
<td>37.38</td>
<td>0</td>
</tr>
<tr>
<td>0.015 mg/l penicillin G</td>
<td>67.36</td>
<td>103</td>
<td>-25.74</td>
</tr>
<tr>
<td>0.03 mg/l penicillin G</td>
<td>30.5</td>
<td>118.08</td>
<td>-14.89</td>
</tr>
<tr>
<td>0.06 mg/l penicillin G</td>
<td>19.04</td>
<td>119.26</td>
<td>-40.36</td>
</tr>
</tbody>
</table>

Fig. 2. Photomicrograph of L. acidophilus ATCC4356. Bacteria are a, thick and spiral; and b, thin and faint-colored from S colonies grown on MRS agar containing 0.015 mg/l penicillin G stained with Gram staining. Scale bar=20 µm.

Fig. 3. Assessment of L. acidophilus ATCC 4356 hydrophobicity in the absence (control) or presence of penicillin G in MRS broth. Error bars represent standard deviations of mean values of results from three independent experiments.

Table 1. Adhesion of L. acidophilus ATCC 4356 under stress (penicillin G) and control conditions.
Effects of penicillin G on physicochemical surface properties

The hydrophobicity was high for the control group, medium in the presence of 0.015 mg/l penicillin G, and low in the presence of 0.03 and 0.06 mg/l of penicillin G.

Thus, the present results showed that the surface properties of L. acidophilus ATCC 4356 were affected by the media composition (i.e., penicillin G). It has also been suggested that the presence of (glycol-) pertinacious material on the cell surface results in higher hydrophobicity, whereas hydrophilic surfaces are associated with the presence of polysaccharides [30]. It is already known that S-layer proteins confer hydrophobicity on the cell surfaces of lactobacilli, implying an ability to adapt the cell surface hydrophobicity in response to environmental changes, such as pH or ionic strength [45]. The experiments of Kos et al. [30] and Schar-Zammaretti et al. [41] also demonstrated a different hydrophobicity value according to the species of lactobacilli and growth medium conditions.

The bacterial adhesion to chloroform and ethyl acetate was tested to assess the Lewis acid–base characteristics of the bacterial cell surfaces. L. acidophilus ATCC 4356 showed a stronger affinity to chloroform, an acidic solvent and electron acceptor, than to ethyl acetate, a basic solvent and electron donor (Table 1) (p < 0.001). Thus, it was concluded that the surface of the bacteria was a strong electron donor.

Production of H$_2$O$_2$ by L. acidophilus ATCC 4356
To investigate the effect of penicillin G on the H$_2$O$_2$ production by L. acidophilus ATCC 4356, the H$_2$O$_2$ concentration was measured in the medium containing penicillin G and the control medium. The results indicated that the H$_2$O$_2$ concentration was highest in the control medium (Fig. 4), whereas the penicillin G treatment decreased the H$_2$O$_2$ production (p < 0.001).

Barnard and Stinson [3] also found that the composition of the growth medium influenced the hydrogen peroxide formation by bacteria.

![Fig. 4. Effects of different penicillin G concentrations on H$_2$O$_2$ production by L. acidophilus ATCC 4356 in MRS broth. H$_2$O$_2$ formation was measured as changes in OD at A$_{655}$ nm. Error bars represent standard deviations of mean values of results from three independent experiments.](image)

S-Protein Extraction and Measurement
The surface proteins of L. acidophilus ATCC 4356 were extracted by treating the whole cells with 4 M guanidine hydrochloride, and then analyzed by SDS–PAGE. A single dominant band of 43–46 kDa, already known as S-proteins [7, 39], and a few faint bands were visible on the gel (Fig. 5A). During the mid-log phase (OD$_{600}$= 0.7), the S-protein production was low in the control group, so no clear 43–46 kDa band was seen on the gel (Fig. 5B, lane S) However, with a higher cell density (OD$_{600}$=0.4), the S-protein production was low in the control group, so no clear 43–46 kDa band was seen on the gel (Fig. 5B, lane S). However, with a higher cell density (OD$_{600}$=0.7), the 43–46 kDa band was clearly visible on the SDS–PAGE gel (Fig. 5A, lane S).

In the presence of 0.03–0.06 mg/l penicillin G, the S-protein band was visible during the mid-log phase.
(OD$_{600}$=0.4), and became sharper with 0.06 mg/l penicillin G (Fig. 5B).

To determine the total proteins, the Bradford method was used. The total proteins (extracted during the mid-log phase) were compared between the control and in the presence of penicillin G. In the case of 0.03–0.06 mg/l penicillin G, the total protein was higher than that in the control, and was further increased when the penicillin concentration was increased (Fig. 6). The total protein production level was lowest with 0.015 mg/l of penicillin G (p < 0.001).

Therefore, it would seem that the S-protein expression was different under unfavorable growth conditions. Khaleghi et al. [26] and Schar-Zammaretti et al. [41] also reported that the composition of the medium can change the S-layer protein expression. Since one of the functions that have been assigned to the S-layer in bacteria is as a protective sheath against hostile environmental agents [7, 41], more S-layer proteins would be expected to be expressed under stressful conditions compared with normal conditions.

Expression of slpA Gene

*L. acidophilus* ATCC 4356 was cultured to the mid-log phase (OD$_{600}$=0.4) in an MRS broth containing 0.015–0.06 mg/l penicillin G and without penicillin G (control). Total RNA was then isolated, reverse transcribed to cDNA, and amplified by a PCR using specific primers for the *slpA* gene. The results indicated that the *slpA* gene expression only decreased with the low concentration (0.015 mg/l) of penicillin G. However, at the higher concentrations of the antibiotic (0.03 and 0.06 mg/l penicillin G), the *slpA* gene expression remained at the same level as that in the control group (Fig. 7) (p < 0.003).

It is unclear why the *slpA* gene expression was similar in the control and 0.03 and 0.06 mg/l penicillin G groups, whereas the S-protein expression was different. It has been suggested that some other genes are expressed in the presence of penicillin G, which may have altered the S-protein production. Altermann *et al.* [1], Kim *et al.* [27], Kristoffersen *et al.* [31], and Lorca and Valdez [33] showed that stress proteins can influence or block *slpA* gene expression, whereas *slpB* is expressed under unfavorable growth conditions instead of *slpA* [9, 10]. Another explanation could be that the S-layer mRNA has a relatively long half-life of 15 min [6] and it can be repeatedly translated.

In conclusion, different penicillin G concentrations were found to alter the colony and bacterial morphotype, surface properties, and S-layer protein and *slpA* gene expressions in *L. acidophilus* ATCC 3456. However, analyzing the expressions of other genes and the effects of stress proteins that may be involved in response to penicillin stress will also improve current knowledge. Yet, whether or not this bacterium will respond to penicillin G in the same way in the gastrointestinal tract environment remains to be clarified.

**Acknowledgments**

This work was supported by the Graduate Studies Office and Research Office of the University of Isfahan and the International Center for Science, High Technology, and Environmental Sciences.

**REFERENCES**


