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# Genetic engineering of UV-mutated *Bifidobacterium longum* and *Lactobacillus acidophilus* in relation to folic acid and Anti-inflammatory productivity

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## Abstract

For folic acid production, two LAB *Lactobacillus acidophilus* (*L. acidophilus*) in addition *Bifidobacterium longum* (B. *longum*) were exposed to UV irradiation for mutagenesis in relation to folate productivity and anti-inflammatory activity.

The results indicated that, fifty mutants were tested towards their folate productivity, four mutants only have productivity higher than Wild strains G5 and K1 resulted from B. *longum* with productivity 68.8 and 56.2% of wild type productivity. Likewise, two mutants of *L. acidophilus* were A2 and C4 with folate productivity 205.2 and 289.3% respectively of Wild type productivity. Results of docking study showed the mode of binding folic acid demonstrated energy binding of of -8.65 kcal/ mol. against DHFR. Folic acid formed four Pi-alkyl, Pi-Pi In addition to Pi-sigma relationships with Ala9, Ile7, Phe34 and Ile60. Additionally, Folic acid interacted with Glu30 and Asn64 through three hydrogen bonds is 1.77, 1.76, In addition to 1.96 Å. The top four UV-mutants was chosen also showed anti-inflammatory activity determined through inhibiting of COX-1 and COX-2 activity higher than both their wild strains and Celecoxib, mutant A2 was the best in inhibiting COX-1 activity at 8.4µg/ml, while mutant C4 was the best in inhibiting COX-2 activity of 0.030 µg/ml. At the level of FolE gene expression, mutant A2 had the highest gene with 1.4fold, followed by mutant K1 with 1.02, then mutant G5 with 0.97 and all these three mutants have expression of gene higher than those of the two genuine strains.

It could be concluded that, *Bifidobacterium longum* and *Lactobacillus acidophilus* were genetically improved with regard to their folic acid productivity and anti-inflammatory activity through UV mutagenesis, that resulted in four mutants have productivity higher than Wild strains. Also, the four mutants have anti-inflammatory activity through inhibiting of COX-1 and COX-2 activity. Molecular docking was performed to confirm theoretically the role of folic acid in DNA synthesis and repair. The results of the docking investigation revealed that the energy binding of folic acid to DHFR was -8.65 kcal/mol. With folic acid, Ala9, Ile7, Phe34, and Ile60 produced four Pi-alkyl, Pi-sigma, and Pi-Pi interactions. Additionally, three hydrogen bonds between folic acid and Glu30 and Asn64, with separations of 1.77, 1.76, and 1.96, were formed.

**Key words**: *FoLE* gene, COX-, COX-2, RT-PCR, DHFR, SDS PAGE.

## 1.Introduction

Dietary folic acid, in particular vitamin B9, is a type of B vitamins that is water soluble. It is an essential nutrient for people. The amino acids change beside synthesis of nucleotide within healthy, proliferating cells are two essential metabolic pathways in which the folate is a cofactor. [1,2,3]. Naturally, folate metabolism abnormality is causally linked to a number of illnesses [4]. Folate insufficiency has been regarded as a crucial component of neural tube malformations, and research has established the effects of folic acid shortage on start defects [5]. Additionally, a higher risk of most malignancies has been linked to folate deficiencies. Low folate balance can also lead to decreased methylation of DNA, that encourages the growth of cancerous cells inside the colon mucosa and supports continuous and rapid epithelial renewal. The European Union has issued a daily consumption recommendation for adults of 400 g/d. [6] Many types of plants, fungi, and bacteria can make folate; however, humans and other mammals are unable to make folic acid [7], thus these co-factors should be obtained from exogenous food sources including fermented dairy products, milk, beside vegetables. Unfortunately, most of the folic acid used for fortification is produced through chemical mean, which may have unintended side effects such protecting against diet deficiency of B12 [8].

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Tetrahydrofolate, 5-formyltetrahydrofolate, and 5-methyltetrahydrofolate (5-MTHF) are significant derivatives of folate produced by microbes [9]. On the other hand, in situ enrichment with folate producing bacteria is a workable solution.

Using Bacterial fermentation for meals is a desired method for growing the folate concentration and dietary cost of foodstuffs. Folate-producing microorganisms are frequently shown in folic acid assay broth. Microbiological assays can be used to determine the overall folate levels of the broths and components used in bacterial fermentation [10]. Folate derivatives can be distinguished using the HPLC-MS method [11].

Lactic acid bacteria (LAB) are taking part withinside the manufacturing of sure health-selling and useful biochemicals together with Folic acid, bacteriocins, natural acids, enzymes, exopolysaccharides, and others for meals and nonfood industries. Many LAB lines had been tested to be able to generate folate, along with *Lactococcus lactis, Lactobacillus plantarum* and *Streptococcus thermophilus* [12]. LAB lines may be viewed as potent probiotics for tolerate folate deprivation if they generate a large amount of folic acid and survive inside that gastrointestinal system. Several LAB lines of folic acid-production by Lactobacillus are currently being tested in fermented food stuffs to increase the folic acid contents [13]. Belongs Fermented milk to the products that is intended to be a substrate with the ability to be fortified with folate [14]. According to studies, combining *Bifidobacterium animalis* and *Streptococcus thermophilus* during the fermentation process of skim milk significantly increased the amount of folate present by more than sixfold. [15]. That although folate kingdom in fermentation process ingredients is because of the existence of LAB lines, it's far nevertheless fantastically low with regards to the endorsed everyday consumption for such an adult (400 g/d).

Techniques using probiotics to improve and modify the balance of the gut microbiota have received a lot of interest recently. Probiotics can modify the gut environment, boost immunity, and provide fitness benefits to the host when given in the right doses [16]. *Lactobacillus plantrum* strain GSLP-7 V was the most effective LAB for this purpose, and beside its fermented yoghurt restored this same important genera like (like as *Dialister, Prevotella, Peptostreptococcus,* in addition to *Streptococcus*), increased serum folate contents in rats lacking in folate [17]. According to earlier research, folic acid-producing LABs established in inside intestine become a potent folate dealer that supplement a folate deficiency[17,18].

The best and most direct option is to test individuals; unfortunately, collecting intestinal microorganisms from human volunteers isn't always morally or financially acceptable [19]. The ability to investigate how probiotic lines interact with that native colonic microbiota, including microbiota population and metabolism, is also provided by in vitro models that replicate the ecology of a human colon (manufacturing of unstable tryptophan derivatives, fatty acids). That importance of probiotics for the human body depends on the well-predicted dose, which is necessary to provide a high-quality effect and should be delivered to the organism through meals in addition to dietary supplements taken as part of a regular diet [20,21].

*Bifidobacterium longum* and *Lactobacillus acidophilus* both have anti-inflammatory properties [21]. Probiotics function through a variety of unique methods [20,22]. They have properties that allow them to interact with the immune system and can modify the digestive tract's flora. To exert dominance over harmful bacteria is one of the most important recognized actions of probiotics. *Lactobacillus* spp. exist in numerous meals merchandise in each a part of the world [23]. They are probiotic microorganisms that may have beneficial effects on a variety of physiological processes, including immune regulation [24]. It also regulates immunity by reducing inflammatory responses via T-lymphocytes [25], B-lymphocytes [26], cells of natural killer (NK) [27], in addition to macrophages [28]. Furthermore, *Lactobacillus rhamnosus* and *L. plantarum* can significantly result in the creation of pro-inflammatory cytokines such as IL-6 and TNF- [29].Several investigators used ultra violet (UV) radiation as bodily device for induction of mutation in prokaryotes cyclobutene pyrimidine dimers and pyrimidine-pyramidone or photoproducts are maximum crucial permutational deoxyribonucleic acid (DNA) lesions brought about through UV radiation. Other lesions, inclusive of DNA strand breaks and thymine glycols also are brought about through UV treatment [30].

Folic acid has a significant impact on the growth and maturation of red blood cells (RBCs). During normal physiological erythropoiesis, folic acid is metabolized to several derivatives in order to prepare for the formation of RBCs. Any folic acid deficiency in the human body causes significant anaemia symptoms. Dihydrofolate reductase (DHFR) has an advantage in folic acid activation because it is a rate-limiting enzyme. In the current work, we attempt to present the interaction between folic acid and DHFR target sites to demonstrate the degree of affinity towards this target. Molecular operating environment (MOE) software was used for the molecular docking process. From the Protein Data Bank, this crystal protein were obtained (PDB) (PDB codes: 1DRF). Therefore, the purpose of this study is to genetically improve the folate productivity of two LAB, namely *L. acidophilus* and *B. longum*, through UV irradiation, in addition to performing a molecular comparison of mutants between them and between wild strains using sodium dodecyl sulfate polyacrylamide gel electrophoresis of

total cellular proteins (SDS- PAGE). Using RT- PCR amplification of this FolE gene, one of the key genes in the examined bacterial cells' folate synthesis pathway, folate productivity in both control strains and four mutants was previously genetically confirmed.

## 2. Materials and Methods

### Materials

### Inoculum preparation

Two Lactic acid bacterial strains *Lactobacillus acidophilus* DSM 20079=ATCC 4356, and *Bifidobacteruim longum* ATCC 15708 were found from the faculty of agriculture at Ain Shams University in Cairo, Egypt's microbial resources center (Cairo MIRCEN) was sub-cultured on de Man , Rogosa and Sharpe (MRS) agar as well as MRS broth media (Oxoid) at $37^{O}$C and 5% $CO_2$ concentration.

### Standard folic acid

5-formyltetrahydrofolate (5-FmTHF), 5-methyltetrahydrofolate (5-MeTHF) , tetrahydrofolate (THF) , in addition to Individual folate standards was purchased from Sigma (St. Louis, MO).

### Human plasma

Sigma sold us human plasma. (St. Louis, MO).

### Experimental procedures

### Preparation of folic acid standard solution

5-formyltetrahydrofolate (5-FmTHF), 5-methyltetrahydrofolate (5-MeTHF) , tetrahydrofolate (THF) , in addition to Individual folate standards was further purified and prepared according to [31**]**.

### Extraction and deconjugation of folic acid

A mixture of 10 millilitres of extraction solution is prepared (0.1 M phosphate buffer with 0.5 % sodium ascorbate) with 6 ml of culture. After 15 minutes in a hot water bath, the mixture was centrifuged at 4000 x g meant for 10 minutes. were added to 3 ml of supernatant along with 0.4 ml of human plasma. Human plasma contains deconjugates that deconjugate the poly glutamine form of folic acid to the mono glutamine form. The mixture was rotated continuously for an hour at a temperature of 37 °C. The reaction were stopped by placing the sample in boiling water for 5 minutes. Extracts were centrifuged for 10 minutes at 27,000 x g. The supernatant were then filtered using a 0.45 µm filter and either used directly or store at −20 °C until use **[32].**

### Ultraviolet Irradiation mutagenesis

The Ultraviolet (UV) irradiation aims to induce desired mutations within genomic DNA of two LAB isolates according to method of [33, 34] with minor modifications where, Overnight cell suspensions were prepared by shaking for 5 minutes. Cells were irradiated through UV radiation for 5, 10, 15, 20 and 30 seconds at 20 cm. The treated suspension kept in the dark and shaded from light for an hour following irradiation. One ml of appropriately diluted treated cells were cultured on MRS agar.

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Fifty mutants were isolated after UV treatment, twenty-five from each strain. All twenty-five mutants were tested for their folate productivity for 24h at $37^{O}$C at concentrations of 2, 3, 4, 5 and 6% $CO_2$ as these mutants may have difference in genetic structure from parental ones.

### Folic acid production by lactic acid bacteria culture

MRS was injected with 2% LAB, then left to incubate at 37 °C. The HPLC method described below was used to assess samples for folate level at 0, 6, 9, 12, 15, and 18 h.

### Analytical methods

### High performance liquid chromatography (HPLC) determination of folic acid

A Hitachi L-6200 HPLC equipment was used for the chromatographic analysis in Tokyo, Japan. The mobile phase were high performance liquid chromatography grade methanol (15%) in 0.05 M KH2PO4. Mobile phases beside samples was filtered via 0.45 µm filters prior to use. used analytical column used was a C18 Hypersil™ (25 cm x 4.6 mm I.D., 5 µm spherical packing: Thermo-Quest, Runcorn, UK). The flow rate through the analytical column was 0.4ml min-1. A fluorescence detector (Hitachi F-1050, Tokyo, Japan) were used (Ex=295 nm, Em=365 nm) **[31].**

### In vitro anti-inflammatory activity

In vitro inhibition assay of cyclooxygenase-1(COX-1) / cyclooxygenase-2 (COX-2) was done using that COX inhibitor screening assay kit (Part No. 560131, CAYMAN Chemicals, MI, USA) that directly measures PGF2 by Sncl2 reduction of COX-derived PGH2 generated in the COX reaction, astragalus polysaccharide (APS) was able to detect COX-1 and COX- was tested for its ability to inhibit 2 was rated. A broad-spectrum antiserum that binds to all major prostaglandin (PG) compounds is used in an enzyme-linked immunosorbent assay (EIA) to measure proteinoid products **[35].**This assay includes both human and recombinant ovine COX-2 enzymes, allowing users to screen for isoenzyme-specific inhibitors. This test is based on PG and PG-acetylcholinesterase (AChE) conjugates competing for his PG antiserum's scarce resources (PG tracer). The ratio of the PG concentration in the well to the amount of PG tracer that can bind to PG antiserum is inverse. This is because the PG concentration is variable, whereas the PG tracer concentration is constant. The rabbit antiserum-PG complexes (free or tracer) in the wells are pre-bound to and bind to this mouse anti-rabbit monoclonal antibody.

Before adding Ellman's reagent (containing AChE substrate) to the wells, wash the plate to remove unbound reagent. The distinctive yellow colour produced by this enzyme process substantially absorbs at 412 nm. The quantity of PG tracer attached to the

wells correlates with the intensity of this hue. It is inversely related to either the bound PG tracer 1/[PG] absorbance or the quantity of free PG that was present in the wells throughout incubation.

**Sodium dodecyl sulphate polyacrylamide gel electrophoresis of whole cell proteins**

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) completed as described by [34] to compare the between wild-type strains and their most potent mutants at total cellular protein. 12% separating gel (pH=8.8) and 4% stacking gel (pH=6.8) were used for electrophoresis, which was carried out at a constant current of 20 mA. In order to photograph, scan, and analyze gels, researchers used the Gel Doc VILBER LOURMAT system.

**Ribonucleic acid isolation and complementary deoxyribonucleic acid synthesis**

**Ribonucleic acid isolation**

Six total ribonucleic acids **(**RNAs**)** were isolated from 72 h LAB strains and their four mutants in liquid MRS medium, used to Trizol (Gibcol) conferring to the manufacturer is protocol. cDNA for the first strand was created using the Advantage RT- for -PCR Kit (Clontech, alo Alto, CA, USA).

**Conventional *reverse transcription polymerase chain reaction* amplification**

*Polymerase chain reaction* (PCR) were performed in 50 µl reactions using primers (**Table 1**) from [36]. A PCR reaction contains of: 100 ng of synthesized cDNA , 200 mM dNTPs , 0.1 mM of each primer , 1.5 mM MgCl2 and one U Taq DNA polymerase (Takara), sterile water to bring the volume to 50 µl. This is the PCR program:

Denaturation for 4 minutes at 94°C, 30 cycles for 30 second at 94°C, 50 second for 56°C, 72°C for one minute, final extension at 72°C for 6 minutes. %TAE buffer [40 mM Tris-Acetate, pH 7.6 - and 1-mM Na-acetic acid (EDTA)] was used to separate agarose gel. Gels were stained with ethidium bromide (0.5 mg/ml) and seen with ultraviolet light after electrophoresis. Sizes were calculated using a standard-length DNA ladder (GeneRulerTM 100 bp DNA Ladder, MBI Fermentans, Vilnius, Lithuania).

**Real time PCR amplification conditions**

Complementary DNA (cDNA) from 6 samples (2 controls and 4 mutants) was subjected to semiquantitative PCR make use of the primers shown in **Table 1**. The first four primers were specific for her FolE gene and the last four primers were specific for her RecA housekeeping was the gene. 12.5 µl of 2x Quantitech SYBR® Green RT Mix (Fermentase. com), 1 µl of 25 pm/l forward and reverse primers, 1 µl of 50 ng cDNA, and 9.25µl of RNase-free water made up the 25 µl of the real-time PCR experiment. Before being loaded into the rotor wells, samples were centrifuged. PCR real-time programmes include: initial denaturation for ten minutes at 95 °C. 40 15-second cycles at 95 °C. 30 seconds of annealing at 59°C and 30 seconds of extending at 72°C. Data gathering done throughout the growth phase. The Rotor Gene 6000 equipment from Qiagen in the USA was used to carry out the reaction. This test employed the 18S ribosomal RNA (rRNA) gene as a housekeeping gene (reference gene). The PCR cycle at which the threshold cycle (CT) results are present.

**Data analysis**

Using the ddΔct technique and Microsoft Excel, comparative quantification analysis was carried out.

**Table 1:** Specific primers used to detect FolE and RecA genes in two LAB strains and their four mutants.

| Primers | Sequence (5'->3') | Template strand | Length | Tm | GC % |
|---|---|---|---|---|---|
| **FW 1FolE** | GCCAGCATAGTCTGAAGTGT | Plus | 20 | 57.61 | 50.00 |
| **RW 1 FolE** | GCAAAAAGCCAACGATGCAAG | Minus | 21 | 59.81 | 47.62 |
| **FW 2 FolE** | TCAACTAGCCGCGGAATTTT | Plus | 20 | 58.18 | 45.00 |
| **RW 2 FolE** | TGGCAGTAGGTGAAGATCCTG | Minus | 21 | 59.17 | 52.38 |
| **FW 1RecA** | GCCCTAATTGGTCCAGGCG | Plus | 19 | 443 963 | 443 981 |
| **RW 1 RecA** | ACAACGGCGTTCTCTCCTAT | Minus | 20 | 444 126 | 444 107 |
| **FW 2 RecA** | ACACAACGTCATTGCAAATGTGA | Plus | 23 | 443 771 | 443 793 |
| **RW 2 RecA** | GCCTGGACCAATTAGGGCAT | Minus | 20 | 443 980 | 443 961 |

**Method of docking**

To prepare for the formation of RBCs, folic acid is metabolized to several derivatives during normal physiological erythropoiesis. Any folic acid deficit in the human body results in significant anemia manifestations. Since DHFR is a rate-limiting enzyme, it has the advantage in folic acid activation. Folic acid and DHFR target sites were specifically chosen to interact in order to demonstrate the high affinity for

this target. The molecular docking procedure was carried out used to Molecular Operating Environment (MOE) software. Protein Data Bank provided the crystal protein (PDB codes: 1DRF).

**Preparation of targeted proteins**

At first water molecules was excluded from the complex. Then, quick preparation were done, missing amino acids was included and unfilled valence atoms was corrected. Protein peptides energy was reduced by applying CHARMM force fields. The protein basic Amino acids selected and prepared for screening; Protein structure was taken from protein data bank.

**Preparation of candidates**

Two-dimensional (2D) structure of folic acid was drawn make use of Chem-Bio Draw Ultra17.0 and saved in spatial data *file* (SDF) format, From MOE Software, opened the saved file , ligand was protonated, and energy were minimized by applying 0.1 RMSD kcal/mol using a MMFF94 force field.

**Docking process**

Docking algorithms was using complete the molecular docking. The ligands could move around freely while the targeted pocket was fixed rigidly. Each molecule was given the opportunity to interact with the protein in twenty distinct ways during the refining process. Then the discovery studio visualizer software generated a 3D orientation and recorded the docking score (affinity interaction energy) of the best-fitted poses with the active site at DHFR.

**Results and Discussion**

**Folate productivity**

First, the two LAB, B. longum and L. acidophilus, were evaluated for their folate productivity, which was found to be 476.1 and 365 g/ml, respectively. Productivity was improved genetically for the two strains through UV mutagenesis for various time periods of 5, 10, 15, 20 and 30 sec, and that surviving ratio in cells was inversely correlated with the length of UV exposure, as shown in **Figure 1**. Results showed that, the exposure to 5% $CO_2$ at 37$^{O}$C was the optimal conditions for folate production by the mutant. The best mutants in their folate productivity from *B. longum* were G5 (198%), K1 (136.3%) of the control one, which was 476.1 µg/ml **(Figure 2).** In case of *L. acidophilus* which gave folate productivity 365 µg/ml (the control), the best producer mutants were C4 and A2 with folate ratio of concentrations 289.3% and 205%, respectively **(Figure 3).**

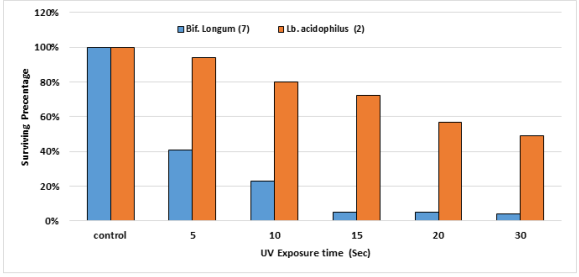



**Figure 1:** Surviving count change (%) of two strains after manipulated by UV irradiation for different times

The best four mutants and their parental two strains were summarized in (**Figure 4). (35)** increased synthesis of B12 and folate but due to EMS mutagenesis in *Klebsiella pneumonia*. The findings demonstrated that all of the chosen mutants are vitamin producers, with Klebsiella pneumonia EMSM33ET emerging as the top isolate. However, when glucose was added to the solid substrate used in the upcoming application and studies, productivity appeared to increase by about three times when compared to the wild strain.

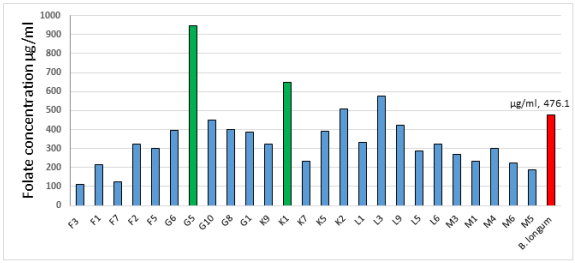



**Figure 2:** The folate productivity (µg/ml) from total isolated mutants as resulted from UV irradiation of *B. longum* strain

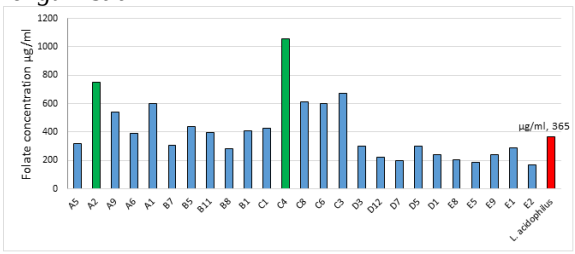



**Figure 3:** The folate productivity (µg/ml) from total isolated mutants as resulted from UV irradiation of *L. acidophilus* strain.

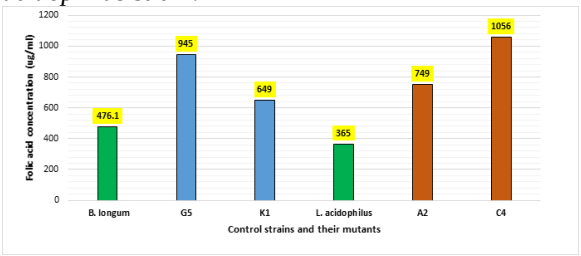



**Figure 4:** Folic acid productivity of two control strains (in green columns), and their four mutants (with blue columns) for B. *longum* (C15708) and (Brown

columns) for *L. acidophilus* (C20079) after 24h of shaking incubator

**Protein levels of COX-1 and COX-2**

Levels of synthesis of COX-1 and COX-2 protein in four mutants were slightly decreased *versus* those of both untreated strains (controls) and Celecoxib. Protein level of COX-1 remarkably decreased in A2 mutant with value of 8.4 *versus* 9.3 µg/ml in wild strain and 13.5 µg/ml in case of Celecoxib. In a different mutant of the same original strain, 8.6 g/ml, it similarly fell but remained marginally higher than those of A2 and C4. While the COX-1 values of the other two *B. longum* mutants, G5 and K1, were greater than those of A2 and C4, they were still lower than those of Celecoxib and their control. They were respectively 9 and 9.2 g/ml (Figure 5). Protein level of COX-2 was decreased less than its value with Celecoxib near to be two times of case of both control strains (*B. longum* and *L. acidophilus*) with values of 0.041 and 0.037µg/ml respectively. Protein values of COX-2 were more decreased when 4 mutants applied to it, where it reached 0.039, 0.037, 0.035, and 0.030 µg/ml with G5, K1, A2 and C4 respectively **(Figure 5).**

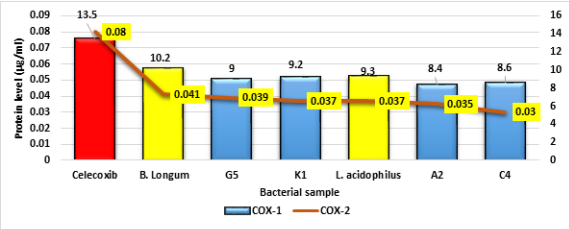



**Figure5:** Protein level of COX-1 and COX-2 anti-inflammatory marker enzymes: COX-1 represented in columns where: red column represents traditional drug Celecoxib; yellow columns represent two LAB strains and blue columns represent the best selected four UV-mutants which resulted from two strains. While COX-2 represented in curve line.

According to research **(38)** *Lactobacillus reuteri* LM1071 showed anti-inflammatory properties by reducing NO generation in lipopolysaccharide (LPS)-stimulated RAW264.7 cells. Depending on the *L. reuteri* population, the expression of iNOS, a gene that produces NO, was also markedly reduced. Furthermore, COX-2 synthesis was inhibited by *L. reuteri* LM1071 at the gene and protein levels. One of the most crucial biomarkers for determining the degree of inflammation in LPS-stimulated RAW264.7 cells is COX-2 **(39, 40).**

**PCR detection and amplification of *FolE* gene**

PCR detection and amplification of *FolE* gene in two control strains and the four mutants was carried out where the amplicons with molecular weight 1400 basis points (bps) in size in agarose gel electrophoresis against Wide Range DNA Ladder 50-10.000 bps **(Figure 6),** where marker lane loaded with 10µl while each sample lane loaded with 15µl of DNA amplicon. Using the RecA gene as a reference gene, RT-PCR was used to assess the expression of the folE gene, which codes for GTP cyclohydrolase I (GCYH-I), a first enzyme in the de novo tetrahydrofolate biosynthetic pathway found in bacteria, fungi, and plants. The expression of the folE gene was higher in four mutants than in control strains, reflecting the high folic acid productivity of these mutants **(30).** Four mutants displayed FLE gene expression folds more than both of their two wild strains when the expression of the FolE gene was measured using folds obtained from the 2^ ddct method; the highest mutant was A2 with 1.46 folds, followed by K1 with 10.3, G5 with 0.97, and C4 with 0.68 folds. **(Figure 7).**

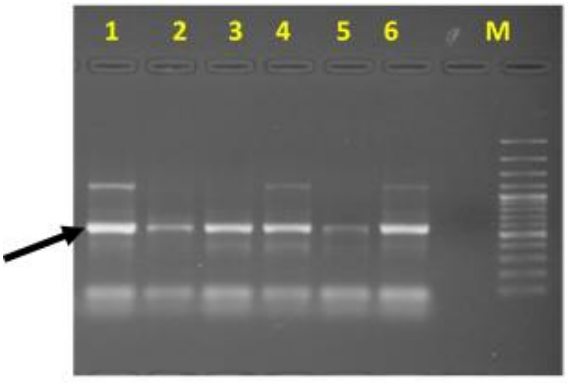



**Figure 6:** Agarose gel electrophoresis of PCR amplification of *FolE* gene from two parental strains and four mutants, bands of gene were estimated nearly 1400 bps as black arrow refers, band (1) mutant A2, band (2) *B. longum*, band (3) C4, band (4) G5, band (5) *L. acidophilus*, band (6) mutant K1, while band (M) DNA ladder

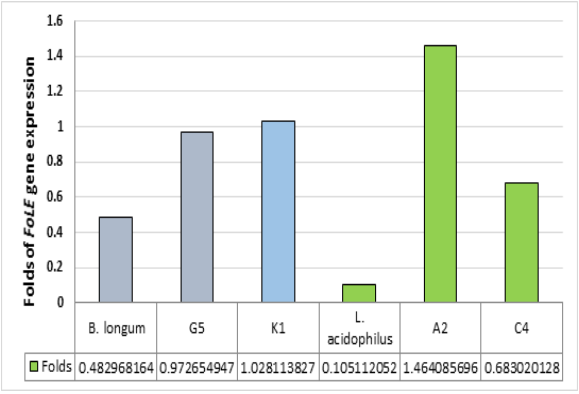



**Figure 7:** Folds of *FolE* gene expression in two wild strains of *B. longum* and *L. acidophilus* beside their best four UV-mutants G5, K1, A2 and C4 after 24 h of fermentation

**Molecular comparison by SDS-PAGE**

Molecular comparison between the best four mutants and their two parental control strains was carried out on the total cellular protein analysis, this achieved through extraction of total protein of these six bacterial samples then performing SDS-PAGE analysis**.**

| Band No | M.W KDa | Tested control and mutants | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 |
| 1 | 215 | 0 | 1 | 1 | 1 | 1 | 1 |
| 2 | 165 | 1 | 1 | 1 | 1 | 1 | 1 |
| 3 | 135 | 1 | 1 | 1 | 1 | 1 | 1 |
| 4 | 84 | 0 | 1 | 1 | 1 | 1 | 1 |
| 5 | 76 | 1 | 1 | 1 | 1 | 1 | 1 |
| 6 | 62 | 1 | 1 | 1 | 1 | 1 | 1 |
| 7 | 54 | 1 | 1 | 1 | 1 | 1 | 1 |
| 8 | 33 | 1 | 1 | 1 | 1 | 1 | 1 |
| 9 | 29 | 1 | 1 | 1 | 1 | 0 | 1 |
| 10 | 23 | 1 | 1 | 1 | 0 | 0 | 0 |
| 11 | 19 | 1 | 1 | 1 | 1 | 1 | 1 |
| 12 | 17 | 1 | 1 | 1 | 1 | 1 | 1 |
| 13 | 15 | 1 | 1 | 1 | 1 | 1 | 1 |
| 14 | `13 | 1 | 1 | 1 | 1 | 1 | 1 |
| 15 | 8 | 1 | 1 | 1 | 1 | 1 | 1 |
| Total | | 13 | 15 | 15 | 14 | 13 | 14 |

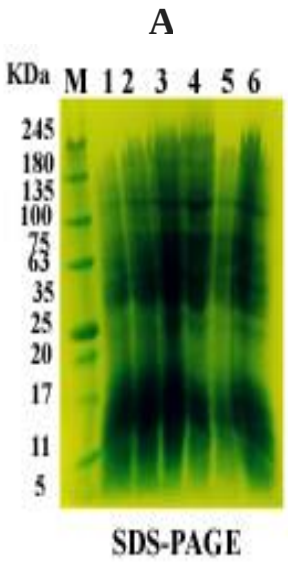



**Figure 8: (A)** SDS-PAGE electrogram of the two LAB strains and the best four mutants within it M (Marker) A2, C4, G5, H2 (*L. acidophilus*), H7 (*B. longum*), K1, **(B)** SDS-PAGE analysis tabulated data of bands (1) A2, (2) C4, (3) G5, (4) H2, (5) H7 and (6) K1

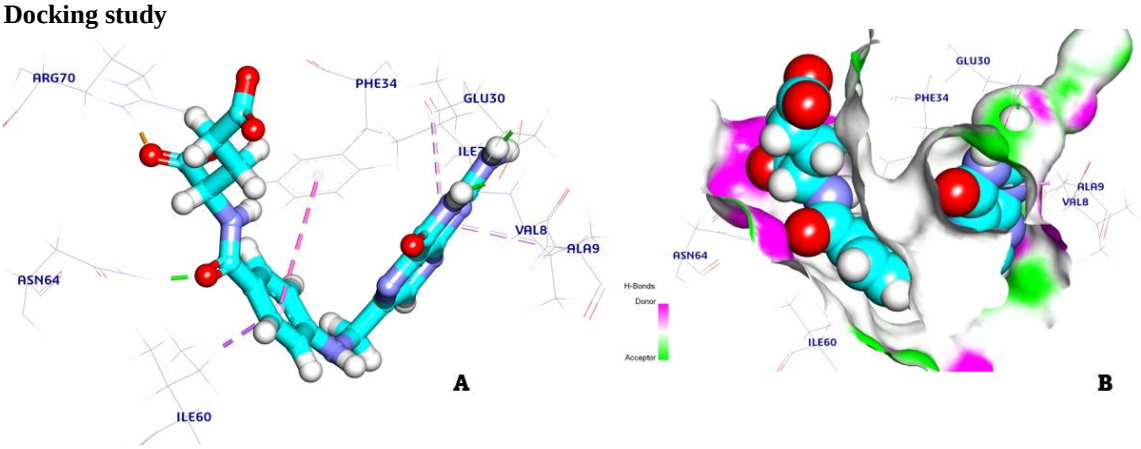
As exhibits in **Figure 8**, SDS-PAGE analysis of four mutants and their two control strains showed protein bands that range from 5 to 245KDa, mutant G5 that represents in band 3 and mutant K1 that represents in band 6 showed total protein bands 15 and 14 respectively which differ from their control that in band 5 with 13 bands. G2 has a single band with size 23KDa. In case of *L. acidophilus* strain which numbered 5 in the order and its two mutants A2 and C4 in order 1 and 2 in gel; mutants A2 and C4 have total protein bands 13 and 15 respectively, while parental strain was 13, A2 and C4 have the single band at 23KDa, whilst the control didn't. A2 also doesn't include bands at 215 and 84KDa while both C4 and control have at these molecular weights.

**Docking study**

Folic acid's binding mechanism demonstrated an energy binding of -8.65 kcal/mol against DHFR. Ala9, Ile7, Phe34, and Ile60 produced four Pi-alkyl, Pi-sigma, and Pi-Pi interactions with folic acid. Moreover, as seen in Figure 9, folic acid connected with Glu30 and Asn64 by three hydrogen bonds that had a distance of 1.77, 1.76, and 1.96.

Table 2: DG, RMSD, interactions (kcal/mol) of Folic acid *versus* targeted sites of DHFR

| Targets screened | Tested compounds | RMSD value (Å) | Docking (Affinity) score (kcal/mol) | Interactions | |
|---|---|---|---|---|---|
| | | | | H.B | Pi – interaction |
| DHFR | Folic acid | 1.14 | -8.65 | 3 | 4 |



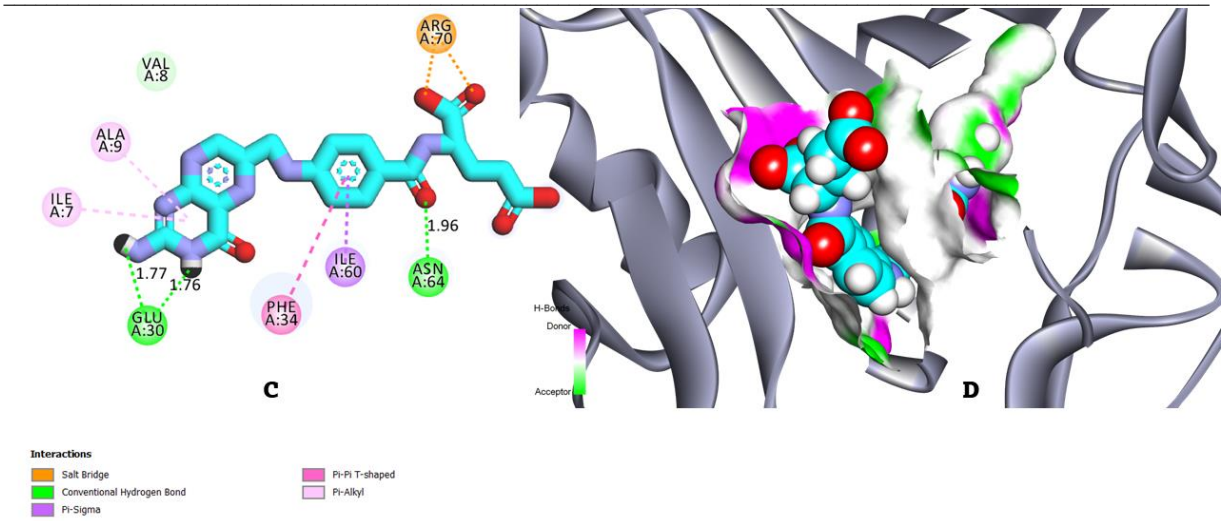



**Figure 9:** Folic acid docked in DHFR, hydrogen bonds in green lines and the pi interactions in pink lines (Pictures A and C) with surface mapping showing Folate occupying the active pocket of DHFR (Pictures B and D)

## Conclusion

UV mutagenesis produced four mutants with productivity higher than wild strains, mutants G5 and K1, with folic acid productivity of 68.8 and 56.2%, respectively, and two additional mutants, A2 and C4, with folic acid productivity of 205.2 and 289.3%, respectively. *Bifidobacterium longum* and *Lactobacillus acidophilus* were genetically improved with regard to their folic acid productivity and anti-inflammatory activity. These four mutants also exhibit anti-inflammatory properties by suppressing COX-1 and COX-2 activity at levels higher than those of their wild-type strains and Celecoxib. Mutant A2 was the best at suppressing COX-1 activity at 8.4 g/ml, while mutant C4 was the best at suppressing COX-2 activity at 0.030 g/ml. Mutant K1 had the second-lowest gene fold of FolE (1.02), while mutant A2 had the highest gene fold (1.4). Theoretically supporting the function of folic acid in DNA synthesis and repair, molecular docking was carried out. Folic acid bound to DHFR with an energy of -8.65 kcal/mol. Four Pi-alkyl, Pi-sigma and Pi-Pi interactions with folic acid were created by the amino acids Ala9, Ile7, Phe34, and Ile60. In addition, folic acid established three hydrogen bonds with Glu30 and Asn64, with separations of 1.77, 1.76, and 1.96.

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