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GENOMICS, TRANSCRIPTOMICS, PROTEOMICS

Non-growth inhibitory doses of dimethyl sulfoxide alter gene expression and epigenetic pattern of bacteria

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Abstract

Although dimethyl sulfoxide (DMSO) is a widely used solvent in scientific research, drug screening settings, and biomedical applications, its solvent (vehicle) effects on biological processes are overlooked. Using *Escherichia coli* as a model, we aimed to investigate and evaluate the effects of low-dose DMSO-driven changes in bacterial cells in a comprehensive and multifaceted manner by combining Fourier transform infrared spectroscopy analyses, analytical cell-biology approaches, and high-throughput sequencing. Here, we show that the non-toxic (1.0 and 2.5%, v/v) DMSO doses reduce the cellular levels of reactive oxygen species, change the cellular nucleic acid content and DNA topology, affect the global 5-methylcytosine pattern of the genome, and modulate gene transcription. These results indicate that even at non-toxic concentrations, DMSO is not inert: it can alter validity by changing or masking the assessed activity of the analyte. Besides, this manuscript does not only highlight that the low, non-toxic solvent doses of DMSO impinge on biological processes, including genome structure and function, but also, the high-throughput sequence data obtained during the study offer a platform for future research to elucidate the mechanism of epigenetically regulated genes in bacteria.

Key points

- *A clear-cut differentiation between the low-dose DMSO-treated and -untreated bacteria by PCA and LDA.*
- *Drastic alterations in the DNA topology and nucleic acids of DMSO-treated bacteria.*
- *Changes in transcriptome and epigenetic signatures with the low-dose DMSO.*

Keywords DMSO · Bacteria · *Escherichia coli* · FTIR · Transcriptome · Methylome

Introduction

Dimethyl sulfoxide (DMSO; C_2H_6OS) is an amphipathic molecule composed of a hydrophilic sulfoxide group and two hydrophobic methyl groups, making the molecule capable of solubilizing polar and non-polar substances and transposing hydrophobic barriers (Brito et al. 2017). DMSO is accepted as a "universal solvent" utilized in biomedical applications and scientific research (Madruga et al. 2017). It is also used as a cryoprotectant to mitigate freezing-related mechanical cell injury (Santos et al. 2003; Jacob and De La Torre 2015).

By describing 20% of DMSO has bacteriostatic properties against *Escherichia coli, Staphylococcus aureus*, and *Pseudomonas bacilli*, the first observations of DMSO as an antimicrobial agent were reported by Jacob et al. (1964). The bactericidal concentration of DMSO required to inhibit growth was determined 40% and 50% for *S. aureus* and *Streptococcus faecalis*, respectively, 30% for *E. coli*, *Enterobacter cloacae*, *Proteus vulgaris*, beta-hemolytic streptococcus group A, and *Salmonella paratyphi B*, 10% for *Pseudomonas aeruginosa*, and 5.0% for *Diplococcus pneumonia* (Pottz et al. 1967). Additionally, Jacob's group observed that 1.0% DMSO enhanced antibiotic sensitivity for antibiotic-resistant tubercle bacillus (Jacob et al. 1964), and 2.5% DMSO, a concentration below the MIC (Minimum Inhibitory Concentration), increased the susceptibility of a multi-drug resistant strain of *Mycobacterium*

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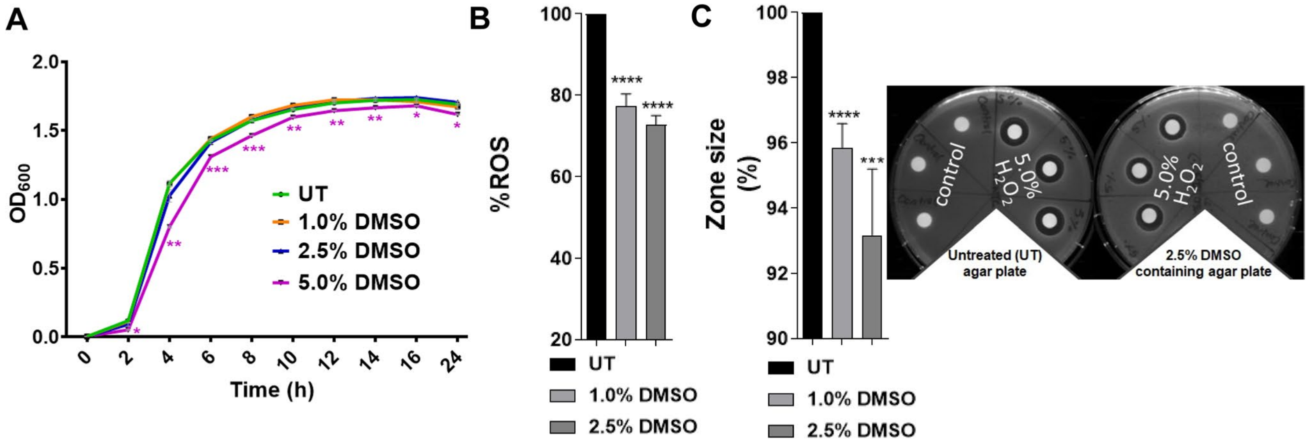


Fig. 1 DMSO showed a ROS-reducing effect in *E. coli*. **A** The bacterial growth was determined at OD_{600} in the presence of 1.0–5.0% DMSO. **B** NBT assay was used to determine the radical scavenging ability of 1.0% and 2.5% DMSO with respect to (%) untreated control (UT). **C** A growth inhibition assay was applied with 5.0% (1.5 M) H_2O_2. The changes in the zone sizes in the presence of DMSO are given on the left. Representative agar plates are shown on the right. In the experiments, the discs embedded with sterilized dH_2O were used as controls (no zone formation). The results are presented as % change compared to untreated control (UT)

tuberculosis to the antibiotic isoniazid (Jagannath et al. 1995): the researchers suggested that these effects could be caused by a DMSO-driven change in the cell wall permeability (Jagannath et al. 1995; Jacob and De La Torre 2015). Jacob and De La Torre (2015) also suggested that DMSO may reduce the pathogenic potential of tuberculosis bacteria through decreasing protein synthesis and reducing energy metabolism, although these assumptions have not been proven experimentally. In another study, 4.0–5.0% DMSO increased the antibacterial effect of ethyl coumarin against pathogenic gram-positive and -negative organisms isolated from burn and wound infections (Hassan 2014). Similarly, Tarrand et al. (2012) showed that DMSO (4.0% and 5.0%) increased the effectiveness of antiseptics, and it was also effective in preventing *Acanthamoeba keratitis* when included in contact lens disinfectants (Siddiqui et al., 2016). These reports show that DMSO can be used as an adjuvant in the prevention of the formation, distribution, and transport of infections. On the other hand, they also propose that DMSO has cellular effects even at doses where it does not have bacteriostatic or bactericidal effects; however, the mechanisms of action have not been described.

While DMSO remains an extensively used vehicle control and widespread solvent/cosolvent in numerous research settings including new drug discovery studies, it is obvious that its influence on biological processes requires further investigation (Sangweni et al. 2021). Guo et al. described that a non-inhibitory dose of DMSO (2.0%) reduced the expression of virulence factors pyocyanin, rhamnolipids, LasA protease, and elastase and inhibited biofilm formation in *P. aeruginosa* under the control of the rhl QS system. Considering DMSO is frequently used to dissolve the test agent in high throughput screening assays for the discovery of QS inhibitors (Guo et al. 2016), it is ironic that DMSO has an impact on the expressions of QS-related virulence factors. Nevertheless, coupled with its apparent low toxicity, the applied vehicle concentrations of DMSO are often unreported due to its obvious and frequent use (Sangweni et al. 2021). Exhibiting the cellular effects of DMSO is needed in terms of understanding the mechanisms beyond the observed solvent actions of DMSO that occur when it is used alone or together with pharmaceuticals. In this study, using *E. coli* as a model, we aimed to explore the low-dose (non-toxic) DMSO-driven changes in bacterial cells in a comprehensive and multifaceted manner.

Materials and Methods

Bacterial Culture and DMSO Treatment

E. coli (ATCC 8739) was grown in Nutrient broth (Nutrient Broth for Microbiology, No. 1, Fluka, Germany) overnight at 37 °C in a 160 × rpm shaking incubator as recommended by the supplied culture collection (https://www.atcc.org/). The optical density of the overnight culture was diluted to OD_{600} of 0.1 before the treatment (1.0%, 2.5%, or 5.0%, v/v) with sterile, cell culture grade DMSO (Santa Cruz Biotechnology, Germany). Control groups (Untreated-UT) were left untreated. To determine the growth dynamics of the DMSO-treated cultures, the optical densities of the cultures at 600 nm were measured every 2 h for 24 h.

NBT (Nitroblue tetrazolium) Assay

NBT is an artificial electron acceptor and the NBT assay is suggested to be a useful tool in the studies of free radicals. NBT assay was applied as described before (Taskoparan

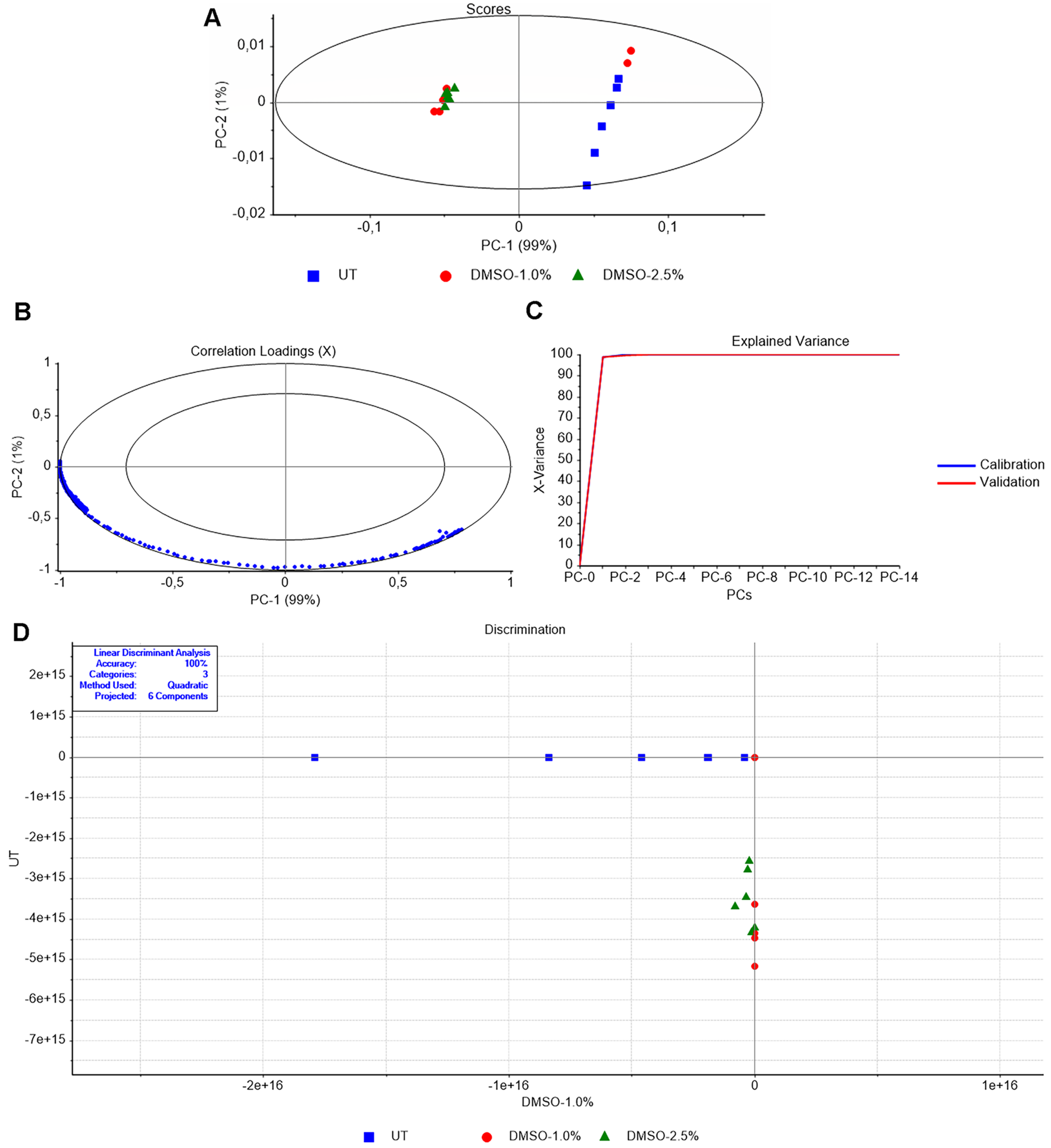
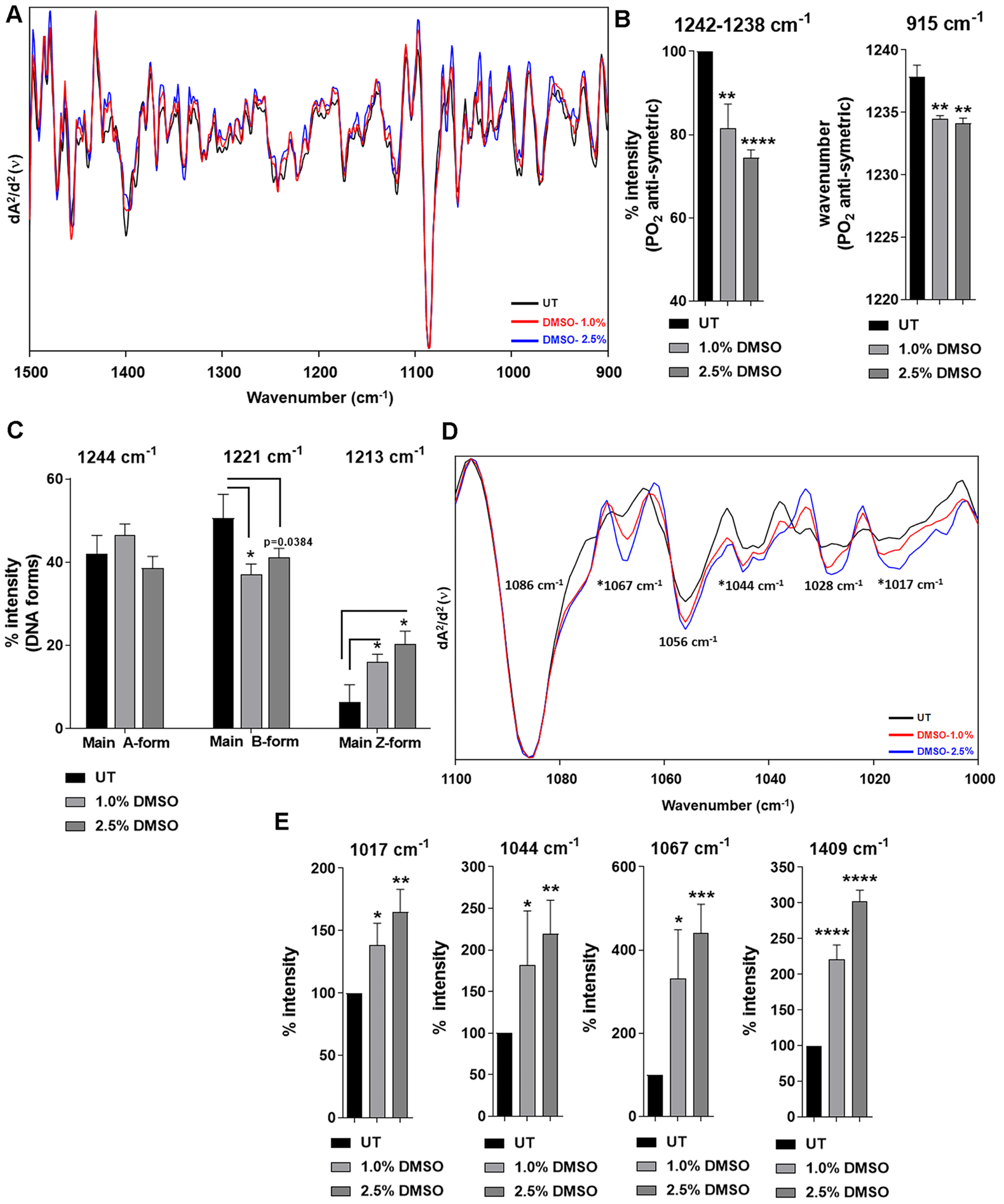


Fig. 2 Spectral data analyses reveal a well separation of low-dose DMSO-treated bacteria from untreated control cells. **A** Scores, **B** correlation loadings, and **C** explained variance plots of the unsupervised PCA model in the spectral range of 1700–650 cm^{-1}. **D** Discrimination plot of supervised LDA model in the spectral range of 1700–650 cm.$^{-1}$

et al. 2017) but with modifications. Please see the Supporting Information for the details.

Zone Inhibition Assay

To determine if DMSO can change the sensitivity to



hydrogen peroxide-mediated killing, a zone inhibition assay was performed as previously described (Kibeom et al. 2020) with some modifications. The details of the method are given in the Supporting Information.

Infrared Spectroscopy Measurements and Pattern Recognition Analyses

Infrared (IR) spectroscopy measurements and PCA and LDA

◂**Fig. 3** IR spectrum indicates DMSO-driven changes in bacterial nucleic acid content and structure. **A** The comparison of second derivative vector-normalized average spectra of DMSO-treated and -untreated control cells (UT) in the spectral range of 1500–900 cm^{-1} is shown. **B** DMSO treatment resulted in a dose-dependent reduction in cellular nucleic acids (left panel) and a shift in the PO_2 wavelength in DMSO-treated cells (right panel). The results are given as % change compared to untreated control (UT). **C** The changes (%) of DNA forms' band intensities as a result of DMSO treatment are shown. **D** The second derivative vector-normalized average spectra in the 1070–1040 $cm.^{-1}$ unique fingerprint region are presented denoting the bands associated with the Z-DNA structure. **E** The changes in the intensities (%) of the Z-DNA associated bands with DMSO treatment are presented compared to untreated control (UT)

analyses were applied as described before (Tunçer et al. 2018). Please see the Supporting Information for the details.

RNA Extraction, RNA-Sequencing, and Bioinformatic and Biostatistics

RNA isolation for RNAseq was performed using the "RNA All-In-One DNA/RNA/Protein Miniprep Kit" (Cell, Tissue, Plant; Bio Basic, Canada) according to the manufacturer's instructions (please see Supplementary Information for the details). Concentrations of the samples and $OD_{260/280}$ and $OD_{260/230}$ values of RNAs were determined using BioSpec-nano (Shimadzu Biotech, Japan) and the samples were visualized on 1.2% agarose gel. All the samples were ensured to meet the desired standards for sequencing ($OD_{260/280}$ ranges from 1.8 to 2.1 and the concentrations 50 ng/μL in a total volume of 25–50 μL).

The RNAseq and bioinformatics analysis were performed by Ficus Biotechnology (Ankara, Turkey). Please see the Supporting Information for the details. The sequence data have been deposited at the Sequence Read Archive (SRA) in the NCBI (https://www.ncbi.nlm.nih.gov/) under the bioproject PRJNA777047. The SRA accession numbers for the untreated control sample, 1.0% and 2.5% DMSO-treated samples are SRR16690734, SRR16690860, and SRR16691093, respectively.

DNA Isolation, Bisulfite Sequencing, and Bioinformatic and Biostatistics

For WGBS (whole genome bisulfite sequencing), DNA isolations from bacteria incubated for 10 h with 1.0% and 2.5%, v/v DMSO or left untreated were carried out using "NucleoSpin Tissue, Mini kit for DNA from cells and tissue" (Macherey–Nagel, Germany), according to the manufacturer's instructions (please see Supplementary Information for the details). The concentration and purity ($OD_{260/280}$ and $OD_{260/230}$ values) of the obtained DNA samples were determined using BioSpec-nano (Shimadzu Biotech) and the samples were also visualized on 1.0% agarose gel. DNA samples meet the desired standards for WGBS ($OD_{260/280}$ 1.8–2.1 and ≥ 100 ng/μL concentration in 50 μL).

The WGBS and bioinformatics analyses of the results were performed by Ficus Biotechnology (Turkey). Please see the Supporting Information for the details. FASTQ Illumina sequence data are deposited at the NCBI Sequence Read Archive (SRA) under the accession numbers SRR16916456, SRR16916842, and SRR18740011 for the untreated control sample, 1.0% and 2.5% DMSO-treated samples, respectively. The BioProject accession number is PRJNA779605.

5-mC ELISA Assay

Colorimetric MethylFlash Global DNA Methylation (5-mC) ELISA Easy Kit (EpiGentek, NY, USA) was used for the detection of the global DNA methylation levels by following the manufacturer's instructions. Please see the Supporting Information for the details of the protocol.

Statistical Analysis

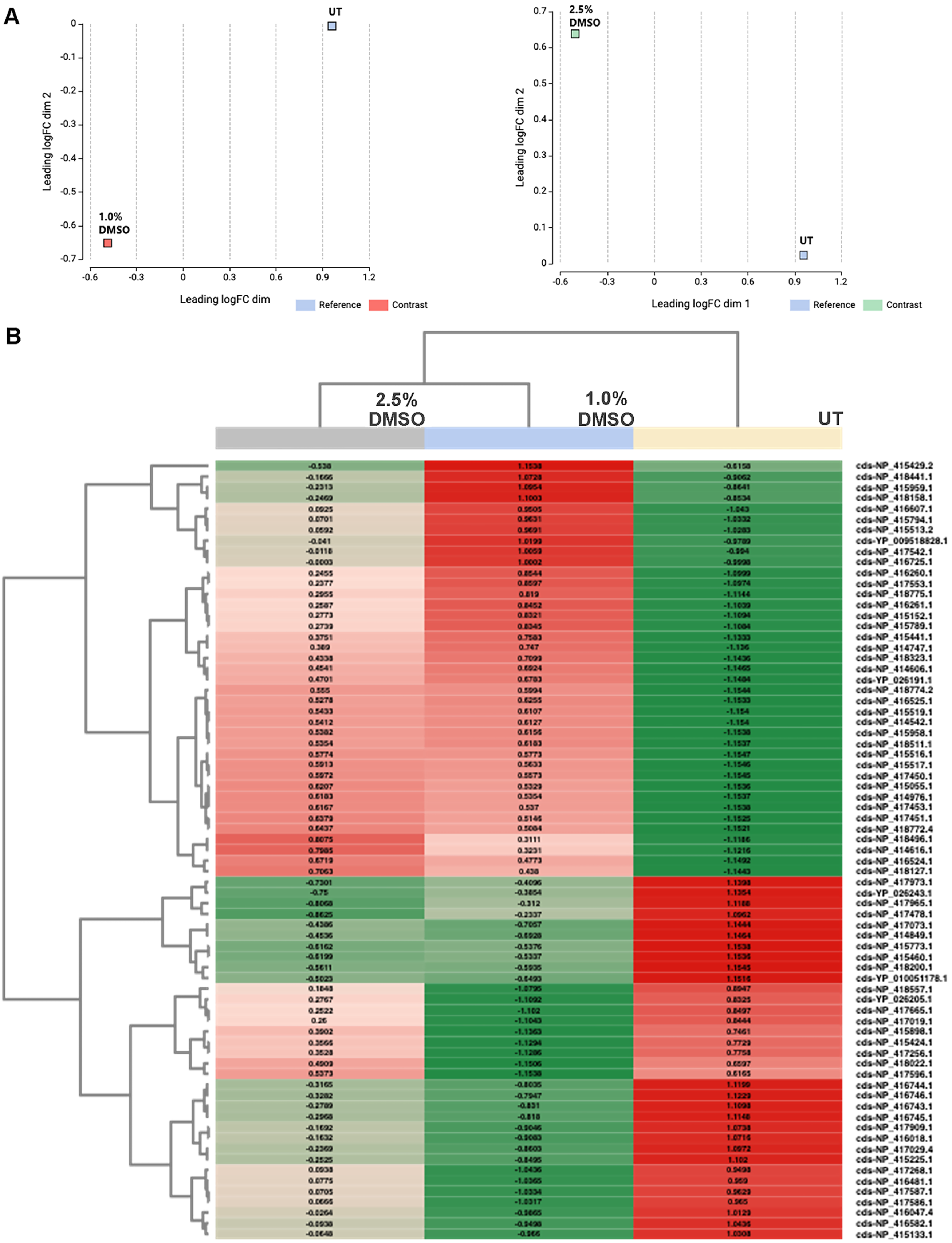
All data are presented as the means ± standard error of the mean (SEM). GraphPad Prism v8 (GraphPad, La Jolla, CA, USA) software was used for the preparation of the graphs and statistical analysis. The experiments were repeated at least three times with at least three technical replicates and *t*-test was used to compare the results with respect to untreated cells. Differences were considered to be significant when the *p*-value was lower than 0.05. The degree of significance was denoted as $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, and $****p \leq 0.0001$.

Results

DMSO Reduces the Formation of Reactive Oxygen Species

To determine the growth dynamics of *E. coli* in the presence of 1.0–5.0% DMSO, the bacteria were cultured for 24 h by measuring the optical densities of the cultures for every 2 h. As shown in Fig. 1A, 1.0% and 2.5% DMSO did not cause a change in bacterial growth rate compared to the control group. The curve also shows the culture enters the stationary phase of the growth after 10 h of incubation. Thus, bacteria were examined in the late logarithmic-early stationary phase (after 10 h of incubation) in all experiments (Militello et al. 2014, 2020).

Several previous studies have shown that DMSO has antioxidant properties (Sanmartín-Suárez et al. 2011; Tunçer et al. 2018). Since Reactive oxygen species (ROS) has been shown to play an important role in antimicrobial lethality (Zhao and Drlica 2014), we examined the ROS-reducing activity of non-growth inhibitory concentrations



2.5% DMSO	1.0% DMSO	UT	
-0.538	1.1538	-0.6158	cds-NP_415429.2
-0.1666	1.0728	-0.9062	cds-NP_418441.1
-0.2313	1.0954	-0.8641	cds-NP_415959.1
-0.2469	1.1003	-0.8534	cds-NP_418158.1
0.0925	0.9505	-1.043	cds-NP_416607.1
0.0701	0.9631	-1.0332	cds-NP_415794.1
0.0592	0.9691	-1.0283	cds-NP_415513.2
-0.041	1.0199	-0.9789	cds-YP_009518828.1
-0.0118	1.0059	-0.994	cds-NP_417542.1
-0.0003	1.0002	-0.9998	cds-NP_416725.1
0.2455	0.8544	-1.0999	cds-NP_416260.1
0.2377	0.8597	-1.0974	cds-NP_417553.1
0.2955	0.819	-1.1144	cds-NP_418775.1
0.2587	0.8452	-1.1039	cds-NP_416261.1
0.2773	0.8321	-1.1094	cds-NP_415152.1
0.2739	0.8345	-1.1084	cds-NP_415789.1
0.3751	0.7583	-1.1333	cds-NP_415441.1
0.389	0.747	-1.136	cds-NP_414747.1
0.4338	0.7099	-1.1436	cds-NP_418323.1
0.4541	0.6924	-1.1465	cds-NP_414606.1
0.4701	0.6783	-1.1484	cds-YP_026191.1
0.555	0.5994	-1.1544	cds-NP_418774.2
0.5278	0.6255	-1.1533	cds-NP_416525.1
0.5433	0.6107	-1.154	cds-NP_415519.1
0.5412	0.6127	-1.154	cds-NP_414542.1
0.5382	0.6156	-1.1538	cds-NP_415958.1
0.5354	0.6183	-1.1537	cds-NP_418511.1
0.5774	0.5773	-1.1547	cds-NP_415516.1
0.5913	0.5633	-1.1546	cds-NP_415517.1
0.5972	0.5573	-1.1545	cds-NP_417450.1
0.6207	0.5329	-1.1536	cds-NP_415055.1
0.6183	0.5354	-1.1537	cds-NP_414976.1
0.6167	0.537	-1.1538	cds-NP_417453.1
0.6379	0.5146	-1.1525	cds-NP_417451.1
0.6437	0.5084	-1.1521	cds-NP_418772.4
0.8075	0.3111	-1.1186	cds-NP_418496.1
0.7985	0.3231	-1.1216	cds-NP_414616.1
0.6719	0.4773	-1.1492	cds-NP_416524.1
0.7063	0.438	-1.1443	cds-NP_418127.1
-0.7301	-0.4096	1.1398	cds-NP_417973.1
-0.75	-0.3854	1.1354	cds-YP_026243.1
-0.8068	-0.312	1.1188	cds-NP_417965.1
-0.8625	-0.2337	1.0962	cds-NP_417478.1
-0.4386	-0.7057	1.1444	cds-NP_417073.1
-0.4536	-0.6928	1.1464	cds-NP_414849.1
-0.6162	-0.5376	1.1538	cds-NP_415773.1
-0.6199	-0.5337	1.1536	cds-NP_415460.1
-0.5611	-0.5935	1.1545	cds-NP_418200.1
-0.5023	-0.6493	1.1516	cds-YP_010051178.1
0.1848	-1.0795	0.8947	cds-NP_418557.1
0.2767	-1.1092	0.8325	cds-YP_026205.1
0.2522	-1.102	0.8497	cds-NP_417665.1
0.26	-1.1043	0.8444	cds-NP_417019.1
0.3902	-1.1363	0.7461	cds-NP_415898.1
0.3566	-1.1294	0.7729	cds-NP_415424.1
0.3528	-1.1286	0.7758	cds-NP_417256.1
0.4909	-1.1506	0.6597	cds-NP_418022.1
0.5373	-1.1538	0.6165	cds-NP_417596.1
-0.3165	-0.8035	1.1199	cds-NP_416744.1
-0.3282	-0.7947	1.1229	cds-NP_416746.1
-0.2789	-0.831	1.1098	cds-NP_416743.1
-0.2968	-0.818	1.1148	cds-NP_416745.1
-0.1692	-0.9046	1.0738	cds-NP_417909.1
-0.1632	-0.9083	1.0716	cds-NP_416018.1
-0.2369	-0.8603	1.0972	cds-NP_417029.4
-0.2525	-0.8495	1.102	cds-NP_415225.1
0.0938	-1.0436	0.9498	cds-NP_417268.1
0.0775	-1.0365	0.959	cds-NP_416481.1
0.0705	-1.0334	0.9629	cds-NP_417587.1
0.0666	-1.0317	0.965	cds-NP_417586.1
-0.0264	-0.9865	1.0129	cds-NP_416047.4
-0.0938	-0.9498	1.0436	cds-NP_416582.1
-0.0648	-0.966	1.0308	cds-NP_415133.1

◂**Fig. 4** Low-dose DMSO treatment affects gene expression. **A** Similarities were visualized among the DMSO-treated samples and untreated control (UT) using 2-dimensional multidimensional scaling (MDS) plots. **B** The heat map depicts the expression profiles of the differentially expressed genes (DEGs) with DMSO treatment compared to untreated control (UT)

of DMSO. NBT (Nitroblue tetrazolium) assay was used to determine the radical scavenging ability of 1.0% and 2.5% DMSO in comparison with untreated *E. coli*. As shown in Fig. 1B, even at low doses, DMSO reduces ROS formation and protected the cells from hydrogen peroxide–mediated killing to some extent (Fig. 1C).

ROS, commonly considered to include superoxide, hydrogen peroxide, and hydroxyl radical, cause several types of intracellular damage and therefore contribute to cell death (Wang et al. 2010; Hong et al. 2019) indicating that when ROS are involved in the lethality, compounds that have radical scavenging or antioxidant properties may interfere with the antimicrobial-mediated killing. For instance, Mi et al. showed that 7.5% (one-half MIC) and 5% (one-third MIC) DMSO protect *E. coli* from rapid antimicrobial-induced cell death through the reduction of intracellular ROS levels (Mi et al. 2016). However, our results suggest that even at non-growth inhibitory doses, DMSO can reduce bacterial ROS formation, and thus, researchers should consider the possibility that DMSO can interfere with the experiment by affecting the antimicrobial activity of test agents.

Infrared Spectroscopy Coupled with PCA and LDA Exhibits a Clear Distinction Between Low-Dose DMSO-Treated and -Untreated Bacteria

The effects of 1.0% and 2.5% DMSO on cellular biomolecules were investigated by attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy. Score and correlation loading plots of principal component analysis (PCA), applied to the spectral data, showed that in the fingerprint region (1700–650 cm^{-1}), DMSO-treated cells were well separated along PC-1 (99%) from untreated control cells (UT) suggesting the occurrence of major biomolecular alterations induced by non-growth inhibitory doses of DMSO (Fig. 2A and B). Explained variance plot fully validated the calibration of the developed model (Fig. 2C). Supervised Linear discriminant analysis (LDA) further confirmed the PCA findings (Fig. 2D): LDA discrimination plots and prediction (Table S1) and confusion matrices (Table S2) show that 1.0% and 2.5% DMSO-treated groups are discriminated from each other and control cells with 100% accuracy.

DMSO Affects Cellular Nucleic Acids

The second-derivative spectra in the fingerprint region (1500–900 cm^{-1}) demonstrated DMSO-induced alterations in nucleic acid–associated bands (Fig. 3A). Therefore, the intensity and wavelength values of the bands specific to certain functional groups of nucleic acids were quantified from the second derivative spectrum.

To determine whether low doses of DMSO affect nucleic acid content, the PO$_2$ antisymmetric band from the absorbance spectrum located between 1242 and 1238 cm^{-1} (assigned to total nucleic acids) and 915 cm^{-1} (ribose loop vibrations in DNA and RNA) were analyzed (Tunçer et al. 2018). As shown in Fig. 3B (left panel), DMSO treatment causes a dose-dependent reduction in cellular nucleic acids. Furthermore, the shift in the PO$_2$ wavelength in DMSO-treated cells indicates DMSO-driven alterations in nucleic acid topology (Fig. 3B, right panel). Based on this result, DNA topological forms and the sub-bands of the PO$_2$ antisymmetric band were analyzed in detail. The A, B, and Z forms of DNA are localized to 1240 cm^{-1}, 1221 cm^{-1}, (Whelan et al. 2011), and 1213 cm^{-1} (Taillandier and Liquier 1992), respectively. The changes in DNA forms are shown in Fig. 3C. From the spectra in the 1070–1040 cm^{-1} unique fingerprint region (Fig. 3D); the increase in Z-DNA as a result of DMSO treatment was also confirmed by analyzing the marker bands associated with the Z-DNA structure: 1017 cm^{-1} (furanose vibration), 1067 cm^{-1} (symmetrical C-O stretch vibration), and 1044 cm^{-1} (antisymmetric C-O stretch vibration) (Banyay et al. 2003; Balla et al. 2016). Finally, the 1413–1408 cm^{-1} region, which is associated with C3-endo deoxyribose found in the Z-form helix (Banyay et al. 2003; Gomes et al. 2009) was analyzed and detected at 1409 cm^{-1}. A significant increase in the intensity of this band, which is expressed as "N-type Z-form," was detected in the samples treated with DMSO (Fig. 3E). Overall, the IR analyses show that DMSO treatment reduces nucleic acid content in bacteria and causes a decrease in the B form and an increase in the Z form DNA, corroborating the previous report revealing the effects of the non-toxic DMSO doses on the nucleic acids of human epithelial cells (Tunçer et al. 2018).

DMSO Changes Transcriptome Profile

Besides having a fundamental role in genetic regulatory mechanisms, DNA topology is also affected by biological processes such as transcription, replication, supercoil and toroid formations, and DNA–protein interactions (Zacharias et al. 1990; Rich and Zhang 2003). Based on the results showing that DMSO alters cellular nucleic acid content and DNA structure (Fig. 3), RNA-seq was conducted to explore differences in *E. coli* transcriptomes in

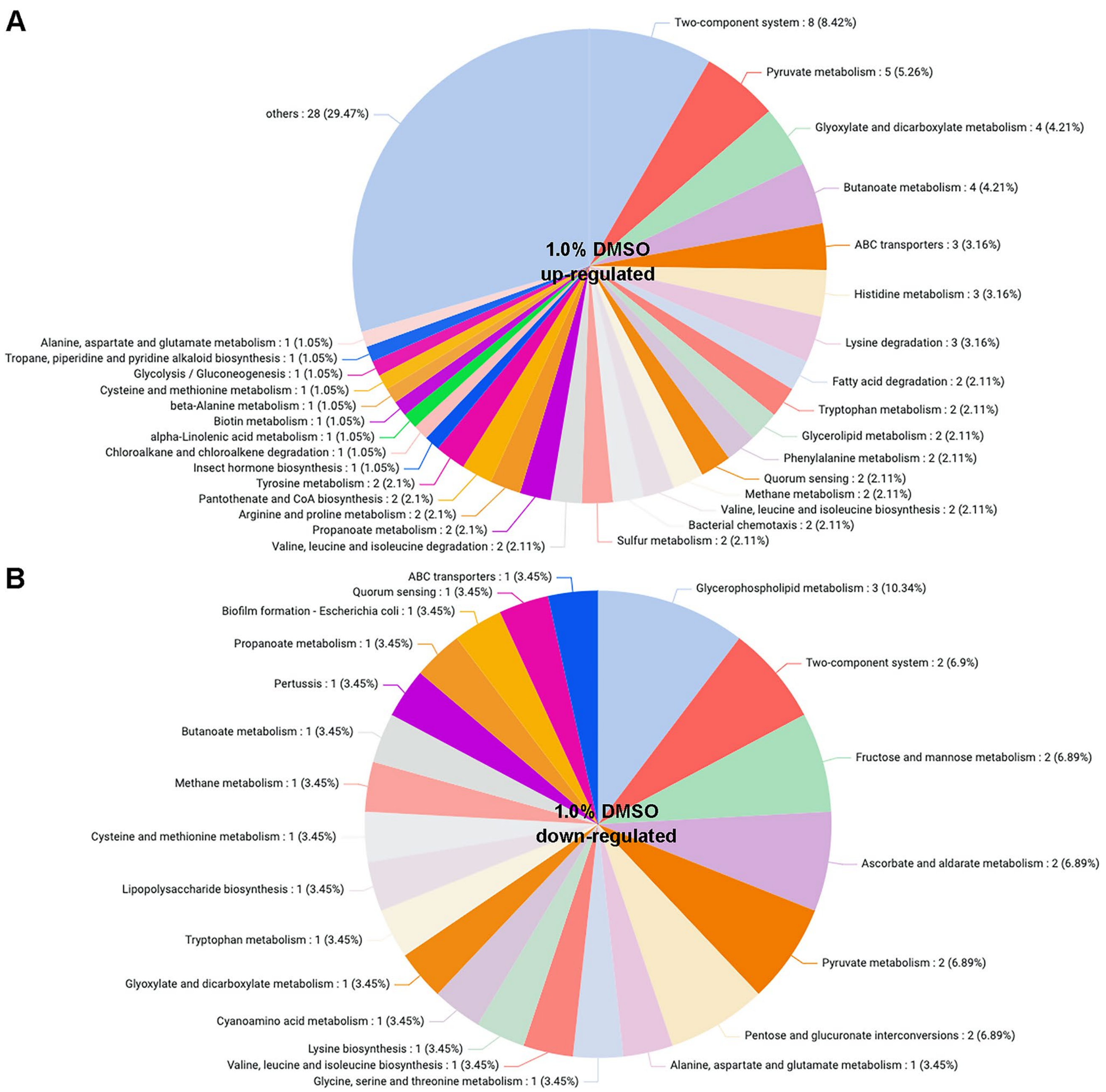


Fig. 5 KEGG pathway enrichment analysis of differentially expressed genes (DEGs) with 1.0% DMSO treatment. **A** Upregulated and **B** downregulated DEGs are presented as pie charts

response to low-dose DMSO treatment. Figure 4A shows the multidimensional scaling (MDS) and Fig. S1 shows mean difference (MD) plots of transcriptome analyses.

By 1.0% DMSO treatment, 39 genes were found to be upregulated ($\log_2$-fold change-$M > 0$) and 34 genes were found to be downregulated ($M < 0$) among 73 differentially expressed genes (DEGs). In a total of 89 DEGs, 52 genes were found to be upregulated ($M > 0$) and 37 of them were downregulated ($M < 0$) with 2.5% DMSO treatment. The genes in which expression was up- or downregulated compared with the untreated control ($p > 0.9$) are given in Tables S3, S4, S5, and S6. The heat maps of the expression profiles of the DEGs upon DMSO exposure are presented in Fig. 4B and the KEGG pathway enrichment analyses of DEGs are given in Figs. 5 and 6. The pathway analyses reveal that low-dose DMSO treatment mostly affects the expressions of the gene function in metabolism. Genes related to the two-component system, the key machinery allowing bacteria to monitor environmental or internal signals and translate these stimuli into appropriate cellular

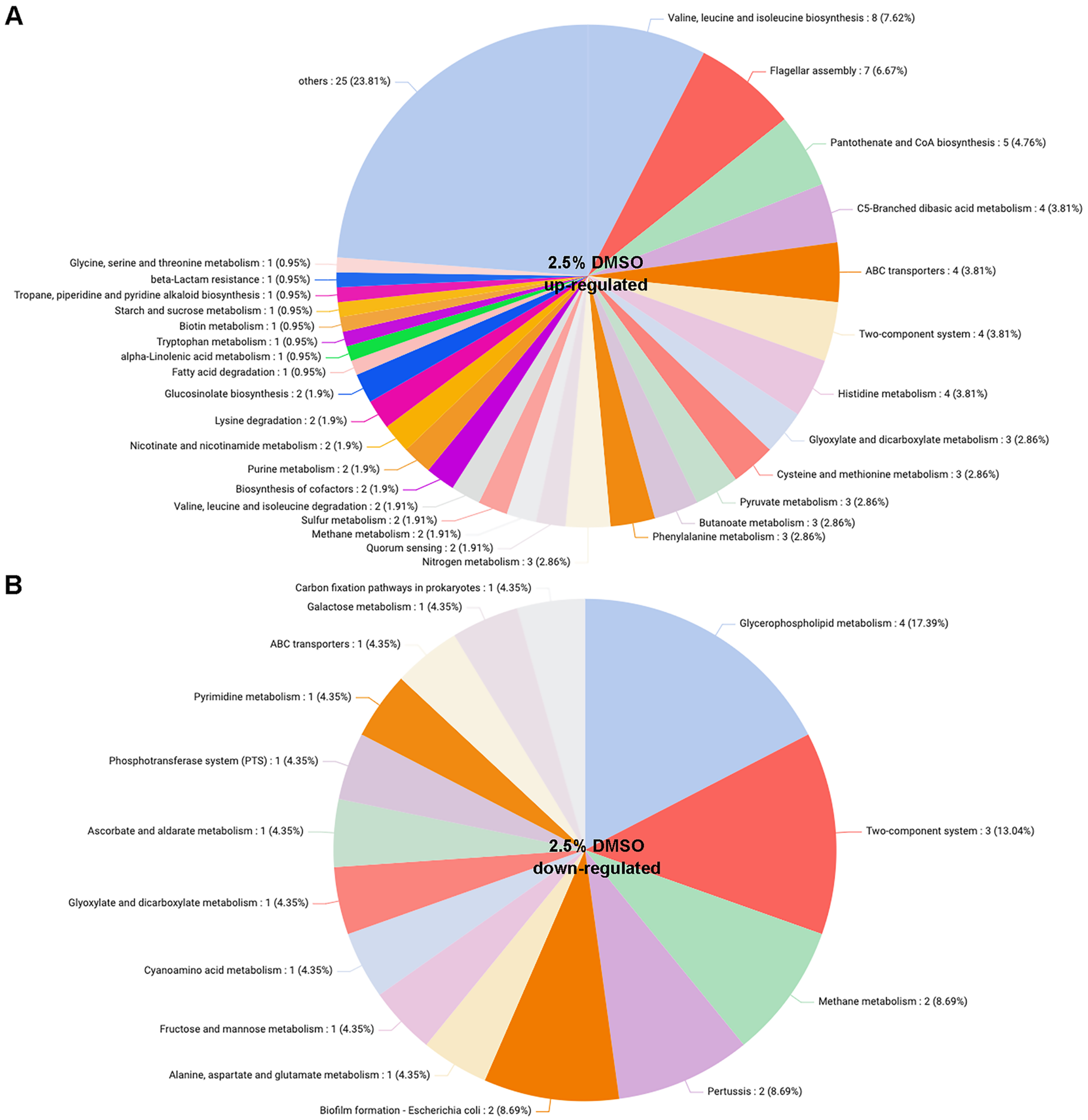
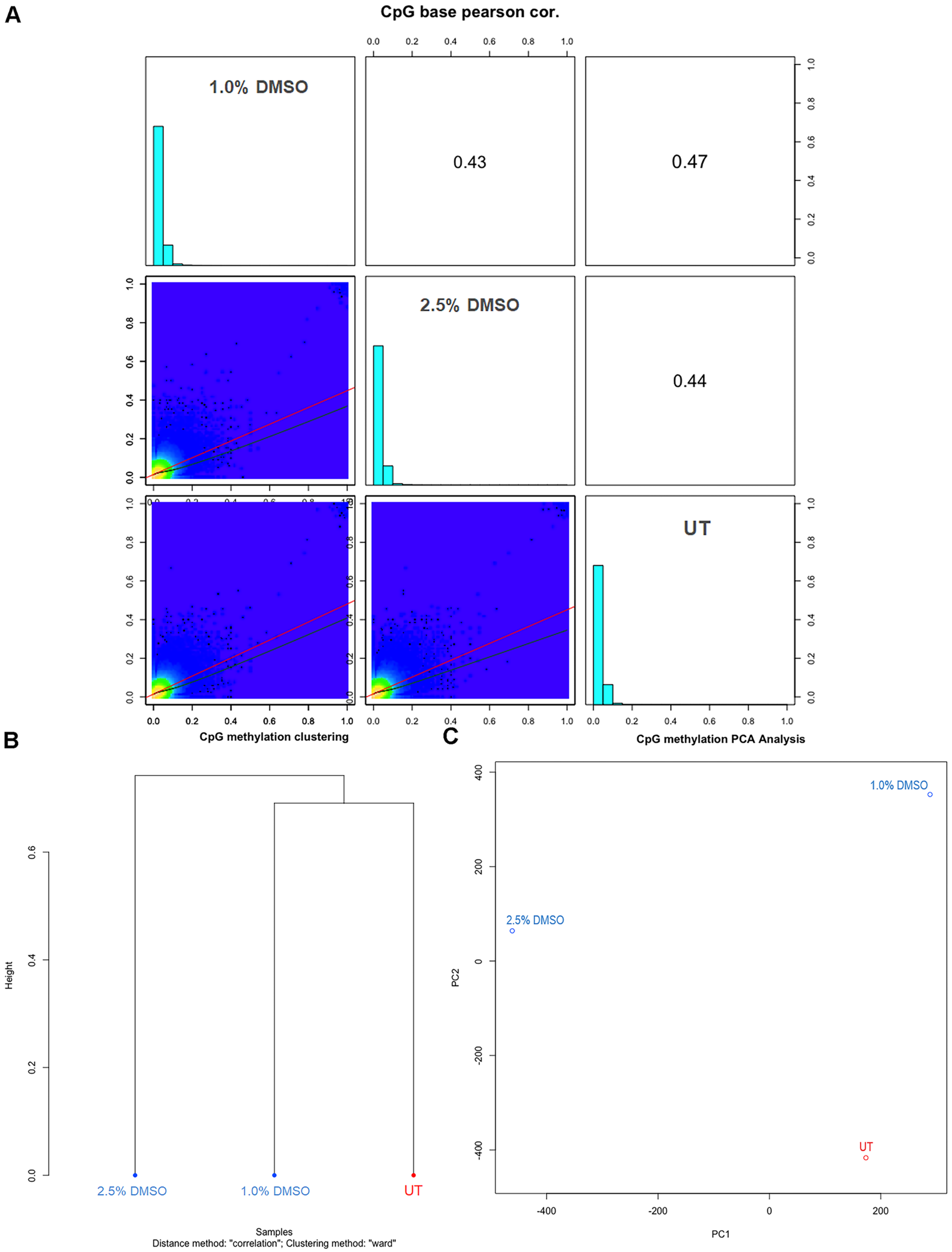


Fig. 6 KEGG pathway enrichment analysis of differentially expressed genes (DEGs) with 2.5% DMSO treatment. **A** Upregulated and **B** Downregulated DEGs are presented as pie charts

responses (Capra and Laub 2012), were also significantly deregulated with the DMSO treatment. Of note, in the level of transcription, the expression of certain genes related to the respiration pathways seem to be differentially regulated by DMSO treatment, such as phenylacetyl-CoA 1,2-epoxidase, reductase subunit (*paaE*), trimethylamine N-oxide reductase 1 (*torA*), cytochrome c menaquinol dehydrogenase (*torC*), and D-lactate dehydrogenase (*ldhA*). Between these genes, the expression of *paaE*, an NAD(P)H oxidoreductase in which the expression is upregulated with DMSO treatment, is referred to as its epoxide and ROS detoxifying activities (Kashyap et al. 2014). Thus, it can be suggested that in addition to its direct ROS scavenging activities (Brayton 1986), DMSO can alter the expression of genes related to oxidative metabolism and therefore can affect the cellular ROS levels and responses to ROS at certain doses.


A
CpG base pearson cor.
1.0% DMSO
0.43
0.47
2.5% DMSO
0.44
UT
0.0
0.2
0.4
0.6
0.8
1.0
CpG methylation clustering
CpG methylation PCA Analysis
B
Height
0.0
0.2
0.4
0.6
2.5% DMSO
1.0% DMSO
UT
Samples
Distance method: "correlation"; Clustering method: "ward"
C
1.0% DMSO
2.5% DMSO
UT
PC2
400
200
0
-200
-400
PC1
-400
-200
0
200

◂**Fig. 7** Sample clustering. **A** The correlation coefficients between pairs of samples are shown as scatter plots of methylation scores. **B** Hierarchical clustering of the samples' methylation profiles using 1-Pearson's correlation distance is shown. **C** PCA on the samples' methylation profiles shows the first two principal components

Gene ontology (GO) enrichment analyses on DEGs were performed as well and the three GO domains, biological process, molecular function, and cellular component, were analyzed for each DMSO treatment dose (Fig. S2). For the genes in which the expressions are modulated, the most significantly over-represented categories in response to both doses of DMSO are (1) "metabolic process," "cellular process," and "response to stimulus" for the GO domain "Biological Process," (2) "cellular anatomical entity," and "protein-containing complex" for the GO domain "Cellular Component," and (3) "binding," "catalytic activity," and "transporter activity" for the GO domain "Molecular Function." Markedly, GO analyses showed that the term "transcription regulator activity," classified under the GO domain of "Molecular Function," is also enriched in the set of downregulated DEGs indicating the impact of the low-dose DMSO treatment on the rate, timing, and/or magnitude of transcription of genetic information (https://www.ebi.ac.uk/QuickGO/term/GO:0140110).

Low-Dose DMSO Treatment Alters Methylation Status of Genes

Several studies indicate that chemical modifications, including cytosine methylation, stabilize the Z-DNA conformation (Rich and Zhang 2003). Zacharias et al. (1990) showed that cytosine methylation enhances the formation of Z-DNA helices at the superhelix density in *E. coli*. Since we have shown that DMSO treatment stabilizes Z-DNA (Fig. 3), we applied 5-methyl cytosine (5-mC) colorimetric ELISA assay and found that DMSO treatment changes the 5-mC status of DNA (Fig. S3). To assess alteration in DNA methylation at base-pair resolution, we performed whole-genome bisulfite sequencing. Histograms of %methylation and read coverage per cytosine for the untreated sample and 1.0% and 2.5% DMSO-treated samples are given in Fig. S4. The similarities between all pairs of samples are given in Fig. 7A as scatter plots of methylation scores in % (Fig. S4). Since correlations above 0.4 are considered relatively strong correlations (moderately strong) (Akoglu 2018), these results reveal that there is a difference in methylation level between the samples. Figure 7B shows the hierarchical clustering using 1-Pearson's correlation distance and Fig. 7C shows the first 2 principal components (PC) from PCA of the samples' methylation profiles. PCA reveals a similar clustering to the hierarchical clustering and both indicate a clear distinction between untreated and low-dose DMSO-treated bacteria. The differentially methylated DNA regions (hypermethylated and hypomethylated) for DMSO-treated *E. coli* with at least a 25% difference and q-value < 0.05 are given in Tables S7 and S8 and promoter and coding region annotations are given in Tables S9, S10, S11, S12, S13, S14, S15, S16, and S17. Finally, the genes in which both the expressions ($p > 0.9$) and methylation status (coding region variants with q-value < 0.05 and $> 25\%$ differences) were changed significantly and differentially with low-dose DMSO treatment are included in Tables S18, S19, S20, and S21.

Previously, it has been shown that DMSO (0.02–2.0%) affects the 5-mC status of genes and intergenic regions in human and mouse cells (Iwatani et al. 2006; Thaler et al. 2012; Verheijen et al. 2019). Here, first time in the literature we show that DMSO has a similar impact on a prokaryotic model. Although enzymatic DNA methylation was reported due to the changes in the increased expression of DNMTs (DNA methyltransferases) (Iwatani et al. 2006; Verheijen et al. 2019), DMSO-driven chemical methylation via methyl radicals cannot be ruled out (Kawai et al. 2010). On the other hand, the observed DMSO-driven hypomethylation (demethylation) can be hypothesized as a result of the antioxidant property (Fig. 1) of the solvent (Beetch et al. 2020; Cui et al. 2022).

In the earlier reports, 5-mC was implicated in the restriction-modification system (RM system) maintenance (Takahashi et al. 2002), phage recombination (Korba and Hays 1982), and transposon insertion (Lee et al. 1987) in bacteria. More recent studies, on the other hand, emphasize the significance of cytosine methylation in bacterial gene expression regulation highlighting its physiological roles. For instance, in *Helicobacter pylori*, the deletion of an orphan C5-cytosine methyltransferase alters the expression of genes involved in motility, adhesion, and virulence (Kumar et al. 2012). Previously, we have shown that C5-cytosine methylation arises as an important aspect of the bacterial adaptation to environmental high-level metal concentrations in *E. coli* and *S. aureus* (Gurbanov et al. 2018) and heavy metal–acclimated freshwater *Gordonia* sp (Gurbanov et al. 2019). In *E. coli*, the conservation of cytosine DNA methylation at 5′CCWGG3′ sequences and *dcm* (DNA cytosine methyltransferase) gene suggests that cytosine DNA methylation has a critical role in *E. coli* biology. Accordingly, Dcm has been shown to have an impact on *E. coli* viability (Militello et al. 2020) and the lack of DNA cytosine methylation has been linked to an increase in the expression of the stress response sigma factor RpoS and many of its targets (Kahramanoglou et al. 2012). Militello et al. also suggested that ribosomal protein gene expression is, at least in part, regulated by DNA methylation in *E. coli* (Militello et al. 2012).

DNA methylation can result in epigenetic regulations by affecting the interaction of DNA-binding proteins with their cognate sites (e.g., steric hindrance) or by changing DNA topology (Payelleville and Brillard 2021). For instance, in the *Vibro cholerae* genome, transcription factors bind to regions lacking 5-mC and prevent methylation (Dalia et al. 2013), implicating a link between 5-mC and transcriptional control of chromosomal genes (Banerjee and Chowdhury 2006). Thus, it can be proposed that 5-mC can influence the sensitivity to an antimicrobial compound through changing gene expression. In fact, *dcm* knockout *E. coli* was found to overexpress the drug-resistance transporter SugE (Militello et al. 2014a). Therefore, one can suggest that DMSO-driven changes in the cytosine methylation profile and the changes in the gene expression can serve for the sustention of bacterial viability as a protection mechanism against toxicity.

Base modifications, including methylation of cytosines, increase the propensity of Z-DNA stabilization (Wang et al. 1984; Xu et al. 2003; Vongsutilers et al. 2020; Kim et al. 2021). Thus, one can suggest that by changing the methylome profile, DMSO treatment can change the DNA topology (i.e., increasing the stability of the Z-DNA regions) (Zacharias et al. 1990). On the other hand, it is also known that DMSO can bind to Z-DNA more strongly than to B-DNA and therefore stabilizes the Z-form (Tunçer et al. 2018). The tight regulation of Z-DNA formation within cells indicates that it plays an important role in cellular activities, including the recruitment of specific proteins/transcription activators or repressors, regulation of gene expression, and control of genome instability (Ravichandran et al. 2019). In the human genome, for instance, DNA regions in the Z-DNA form are associated with active transcription by causing DNA to become looser (Shin et al. 2016) and in *E. coli*, it is the negative supercoiling that induces the formation of the Z-structure of alternating CG that blocks transcriptional elongation (Peck and Wang 1985). Hence, it can be propounded that DMSO-driven changes in DNA topology, as well as cytosine methylation, modulates the transcription.

Discussion

FDA classified DMSO as a class 3 solvent, which is the safest category with a low toxic potential for humans at levels normally accepted in pharmaceuticals (Verheijen et al. 2019). However, we and others have shown that non-growth inhibitory doses of DMSO cause structural and functional changes in human cells (Tunçer et al. 2018; Verheijen et al. 2019; Sangweni et al. 2021). Later on, Costes et al. described how DMSO, when used in concentrations that do not inhibit growth, germination, or sporulation, affects the physiology, transcriptome, and secondary metabolism of fungi (Costes et al. 2021).

In bacteria, the antimicrobial actions of DMSO are suggested as a result of its membrane penetration and perturbation effects (Singh et al. 2021). The effect of DMSO on the structure and properties of cell membranes can occur even at low concentrations, as a result of dehydration of the membrane surface (Dyrda et al. 2019). Here, first time in the literature, we investigated the biological effects of the non-toxic solvent doses of DMSO on bacteria showing that this organosulfur compound has significant effects on nucleic acids: it changes the cellular nucleic acid content, DNA topology, and methylation profile of the genome. Furthermore, in accordance with the previous studies indicating that many small molecules mediate bacterial transcriptional responses at sub-inhibitory concentrations (Goh et al. 2002; Yim et al. 2011), the results of this study reveal that non-growth inhibitory doses of DMSO cause significant alterations also in the transcriptome.

The observed changes can address many of the applications of DMSO in cell biology as well as call attention to some of the hitches when interpreting the study outcome where DMSO is used as a solvent/cosolvent. Assuredly, the data presented here can also serve DMSO to be meticulously investigated for broadening its use in applied science. Using DMSO treatment as a driver, this study also allows analyzing the changes in the gene expression and methylation profile, concomitantly, and comparatively. The roles that epigenetic regulatory systems play in bacterial biology are much less known compared to eukaryotes (Casadesus and Low 2006). Although the data on the bacterial methylome is progressively reported utilizing the advances in long-read sequencing technologies, still there is a gap between the scarce reports of bacterial gene regulation associated with DNA methylation (Payelleville and Brillard 2021). Since methylomic data in bacteria is extremely necessary for elucidating epigenetically regulated genes, this study will also be useful for identifying new candidates for epigenetically regulated genes in bacteria.

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Author contribution ST designed the study and carried out the experiments and analyzed the results, except the FTIR experiments which was performed by RG. ST wrote the manuscript with input from RG. The final manuscript has been read and approved by both authors.

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Data availability All data generated or analyzed during this study are included in this published article and its supplementary information files.

Declarations

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare no competing interests.

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