

The Effect of Sodium Benzoate and Nisin on Human HepG2 Cell Gene Expression

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Abstract

Background and Objective: Sodium benzoate is known as a preservative compound with a high safety profile in the food industrial and pharmaceutical field due to its antibacterial and antifungal properties. In the other hand, nisin is a bio-preservative agent. Determining the effect of sodium benzoate on human HepG2 cell gene expression in comparison with nisin is the aim of this study.

Material and Methods: The effect of sodium benzoate on gene expression of human HepG2 cells was extracted from the Gene Expression Omnibus (GEO) database. Pre-evaluation analysis via GEO2R confirmed valid analysis. The significant differentially expressed genes (DEGs) were investigated via protein-protein interaction (PPI) network analysis and the hubs were screened via a directed regulatory network. The critical hub genes were identified and discussed. The apoptosis-related dysregulated genes of human HepG2 from the literature were assessed among the significant DEGs of sodium benzoate analysis.

Results and Conclusion: A total number of 11521 significant DEGs were identified. The PPI network including 4095 recognized DEGs with 208 hub nodes was created. The hubs were assessed via a directed protein network and MAPK1, CCND1, MAPK14, RAF1, KRAS, MAPK3, PIK3CA, SIRT1, EGF, RBX1, FYN, and NIP7 were pointed out as the critical hub genes in response to presence of sodium benzoate. A total of 78 dysregulated genes of nisin analysis (except TNRSF25) had no common genes with significant DEGs of sodium benzoate evaluation. It can be concluded that sodium benzoate and nisin affect essential cellular functions such as cell cycle progression, cell motility and metabolism, cell proliferation, and survival in various manners. It seems wide usage of food preservative agents requires more investigation to guarantee human health maintenance.

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1. Introduction

Sodium benzoate is known as a preservative compound with a high safety profile in the food industry [1]. It is used widely as an antibacterial and antifungal preservative in food and pharmaceutical fields. It has seen some tender of

sodium benzoate as a therapeutic compound to treat medical disorders [2, 3]. Benzoate teratogenic and neurotoxic effects on zebrafish, chromosome abnormalities in cultured human lymphocytes, the reaction of benzoate with ascorbic acid to



yield carcinogen benzene in drinks, and the effect on neurotransmission and cognitive functioning are the reported disadvantages of benzoate function [4]. Application of sodium benzoate is limited to 0 – 5 mg/kg by the World Health Organization and Food and Agriculture Organization of the United Nations [5]. Investigation indicates that sodium benzoate affects gene expression in the liver. Based on this study various doses of sodium benzoate silenced MAPK8 expression [6].

The growing request for fresh-like foodstuffs regarding the potential health hazards of chemical food preservative compounds led to the rise of food biotechnology features of biological antimicrobial reagents as food preservative tools. Bacteriocins are alternative food preservative substances that are small polypeptide molecules with antimicrobial activity [7]. Nisin is an antimicrobial peptide that was originally detected in milk fermentation culture. It is suggested that bacterial blockage in human foods is a safe agent [8-10]. Nisin as a natural food preservative compound is used in some food products versus many Gram-positive microorganisms. It can be applied alone or with other food preservative compounds [11].

Gene expression analysis appears as a suitable tool to explore the molecular mechanism of diseases and the function of drugs and chemical compounds [12]. Since gene expression analysis products are a set of significant up and downregulated DEGs, bioinformatics is a useful method to evaluate the findings [13]. PPI network analysis as a bioinformatics approach has attracted the attention of researchers to analyze genomic data [14]. The nodes of a PPI network are connected via the edges to form an interactome. In the scale-free PPI network, there are central nodes that are known as hubs. The hub nodes are characterized by a high value of degree. The degree is the number of the first neighbors of a node. It is reported that the hub nodes of a PPI network play critical roles in the function of the studied sample [15, 16]. The directed PPI networks are constructed from nodes that are connected by the directed edges from the causal genes to the affected genes [17]. The activation, inhibition, and expression actions are used in the directed PPI network to explore the critical genes of the studied network [18].

In the present study, gene expression profiles of human HepG2 cells in the presence of sodium benzoate is analyzed via directed PPI network to find the critical affected genes in response to the applied chemical preservative compound. The dysregulated genes of cells in the presence of nisin as a bio-preservative agent are investigated in the literature. Comparison of gene expression alterations of human HepG2 cells in the presence of sodium benzoate and nisin is the main aim of this project.

2. Materials and Methods

2.1. Data collection

The effect of sodium benzoate on gene expression of human HepG2 cells was searched in the GEO database. Information about four samples of treated cells with 10 mM sodium benzoate for 24 hours before harvesting relative to the four individual controls was found in GSE108469 (GPL16791) (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE108469>). Data are retrieved via library strategy; RNA-Seq, library source; transcriptome, library selection; cDNA, and instrument model; Illumina HiSeq 2500. The effect of nisin on gene expression of human HepG2 cells was extracted from published documents of Zainodini N et al [10].

2.2. Pre-evaluation analysis

Data was assessed via the GEO2R program to find the significant DEGs. A Uniform Manifold Approximation and Projection (UMAP) plot was applied to detect the separation of treated cells from the control individuals. Significant up and downregulated DEGs were visualized via volcano plot. A number of significant DEGs relative to the insignificant DEGs were shown via the Venn diagram.

2.3. PPI network analysis

The significant DEGs were investigated via undirected PPI network analysis. The queried significant DEGs were included in the STRING database via “protein query” and the recognized genes were included in the PPI network by Cytoscape software v 3.7.2. The network was analyzed by the “Network analyzer” application of Cytoscape software to find the central nodes. The hubs as central nodes were selected based on degree cutoff = mean + 2(standard deviation). To explore critical genes, hub nodes were assessed via directed PPI network analysis. The hubs were connected by activation, inhibition, and expression links. Since the out-degree value is corresponded to the actor role of a node, the nodes of the network were arranged based on the out-degree value to find the controller DEGs.

2.4. Comparative study

The dysregulated genes of the human HepG2 cells in the presence of nisin were investigated among the reported DEGs of sodium benzoate analysis.

2.5. Statistical analysis

The significant DEGs were determined based on adjusted P-value < 0.05. The PPI network was constructed based on a confidence score = 0.4. Hubs were identified via degree value cutoff = mean + 2(standard deviation).



3. Results and Discussion

3.1. Pre-evaluation analysis

Data was assessed to find the critical significant DEGs that discriminate the treated cells with sodium benzoate from controls. UMAP plot is shown in Figure 1, the treated cells are completely separated from controls. UMAP is applied to reduce dimensions of data to 2-dimensional presentation and the amounts of two axes are not meaningful. The volcano plot (Figure 2) indicates that there are huge numbers of significant DEGs. As depicted in Figure 3, there are 11521 significant DEGs including up and downregulated genes.

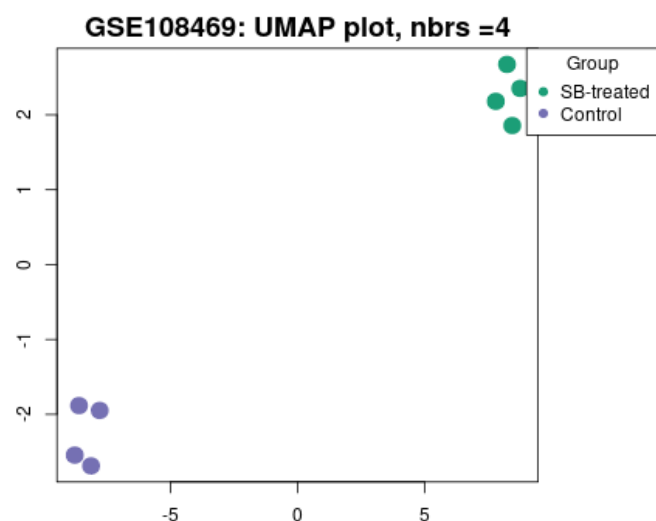


Figure 1. UMAP plot of the treated cells with sodium benzoate versus controls. Nbrs=4 indicates the number of compared neighbors to find similarity for a sample.

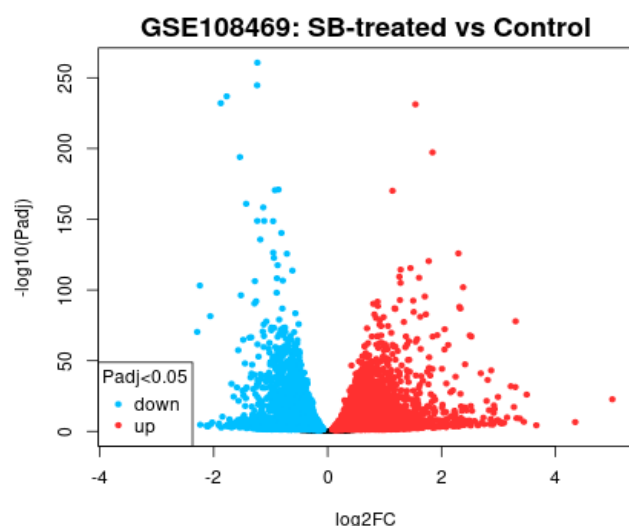


Figure 2. Volcano plot of gene expression comparison between treated HepG2 cells with 10 mM sodium benzoate for 24 hours before harvesting and the control individuals.

GSE108469: DESeq2, Padj<0.05

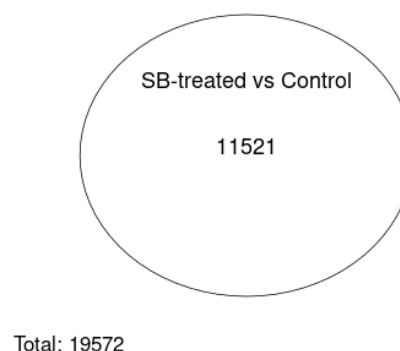


Figure 3. Venn diagram of gene expression analysis of treated HepG2 cells with 10 mM sodium benzoate for 24 hours before harvesting versus the control individuals.

3.2. PPI network analysis

The significant DEGs were included in an undirected PPI network; the interactome was formed from 4095 nodes and 63934 edges. The 208 hub nodes were determined based on degree value cutoff = 101. The hub nodes were inserted in a directed PPI network including 95 isolated genes, 2 paired DEGs, a subnetwork of 13 genes, and a main connected component of 98 nodes. The subnetwork of 13 genes and the main connected components that are connected via activation, inhibition, and expression relationships are shown in Figure 4. For better resolution, the directed PPI network including activation and expression, activation, expression, and inhibition are presented in Figures 5-8 respectively. To find a critical controller hub, the directed PPI network including a subnetwork of 13 genes, and a main connected component of 98 nodes was analyzed and arranged based on out-degree value (see Figure 9).

3.3 Nisin-related gene expression analysis

The up and down-regulated genes in the presence of nisin which are extracted from the document of Zainodini N et al [10] are shown in Table 1. As depicted in Table 1, 78 dysregulate gene are appeared as targeted genes by nisin. It should be mentioned that the dysregulated genes were identified based on the human apoptosis PCR array to find genes activated by nisin during possible apoptosis. This method has limited the presented data to apoptosis.



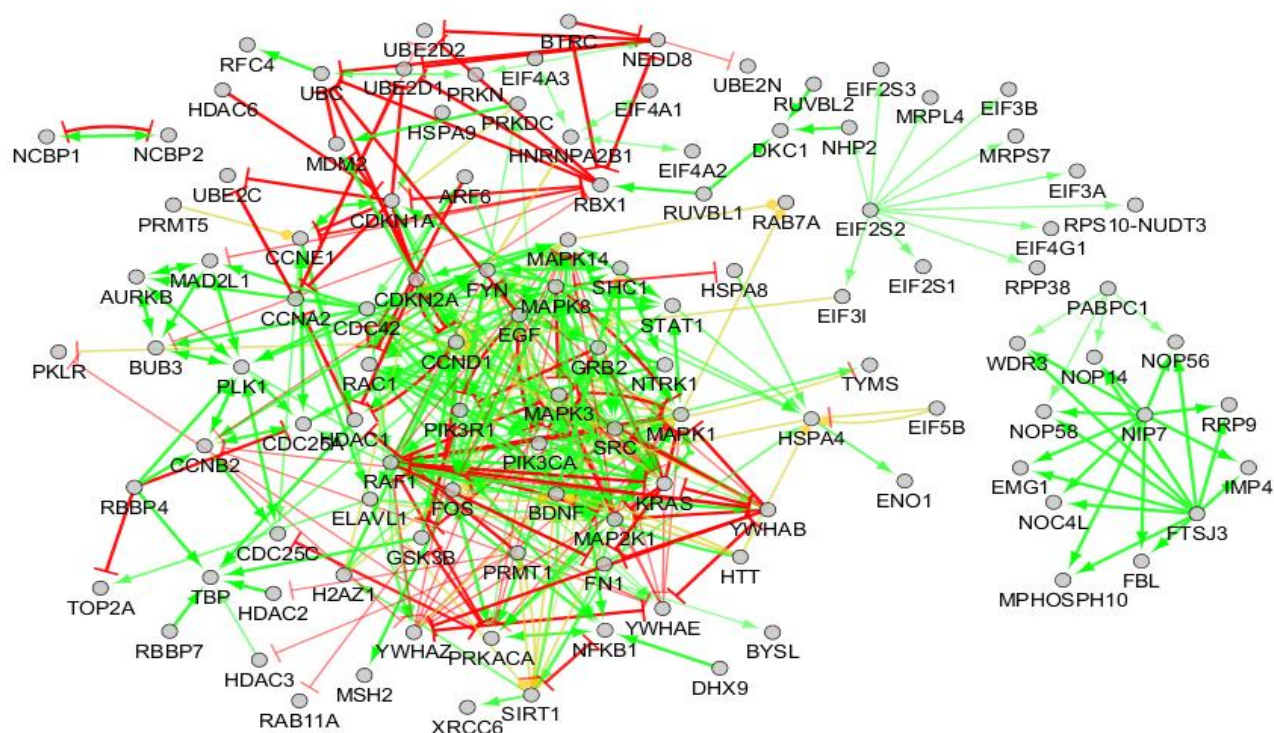


Figure 4. A subnetwork of 13 DEGs and the main connected component of 98 nodes related to the directed PPI network of the hubs of the undirected PPI network. Green, red, and yellow refer to activation, inhibition, and expression respectively.

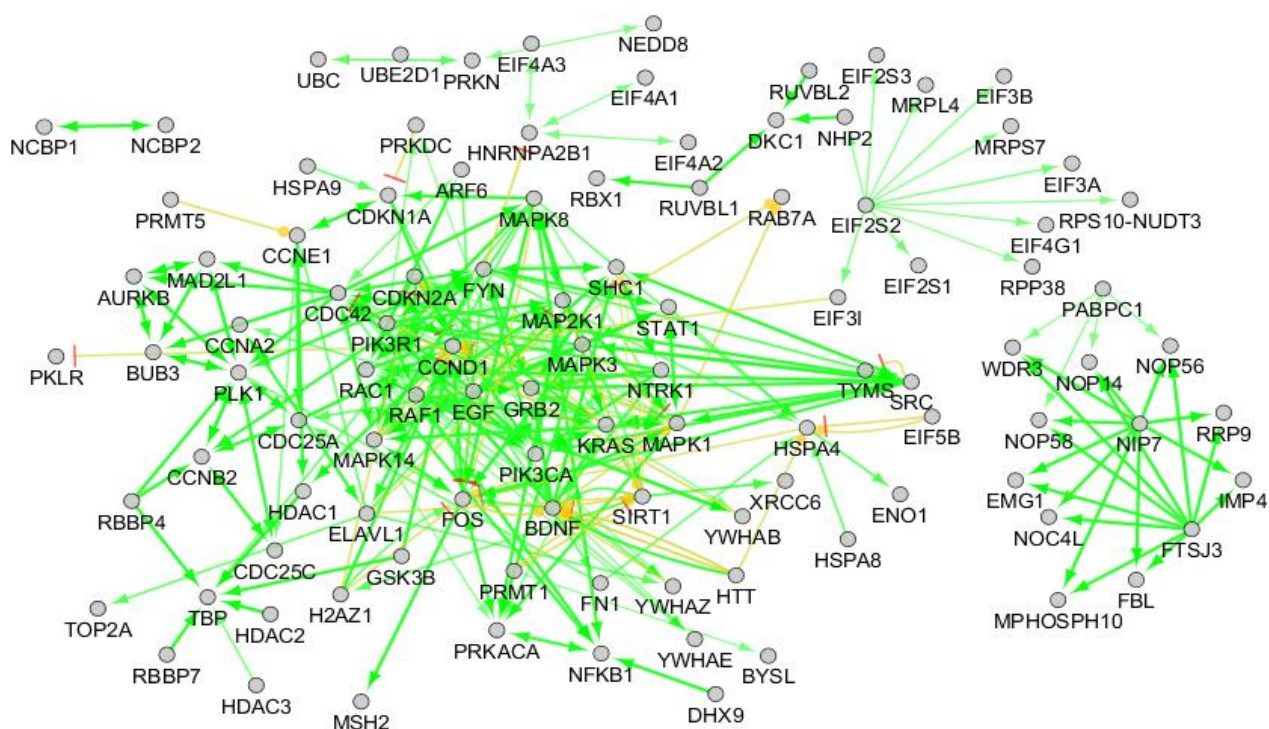


Figure 5. Directed PPI network of the hubs of the undirected PPI network including activation and expression connections. Green and yellow refer to activation and expression respectively.



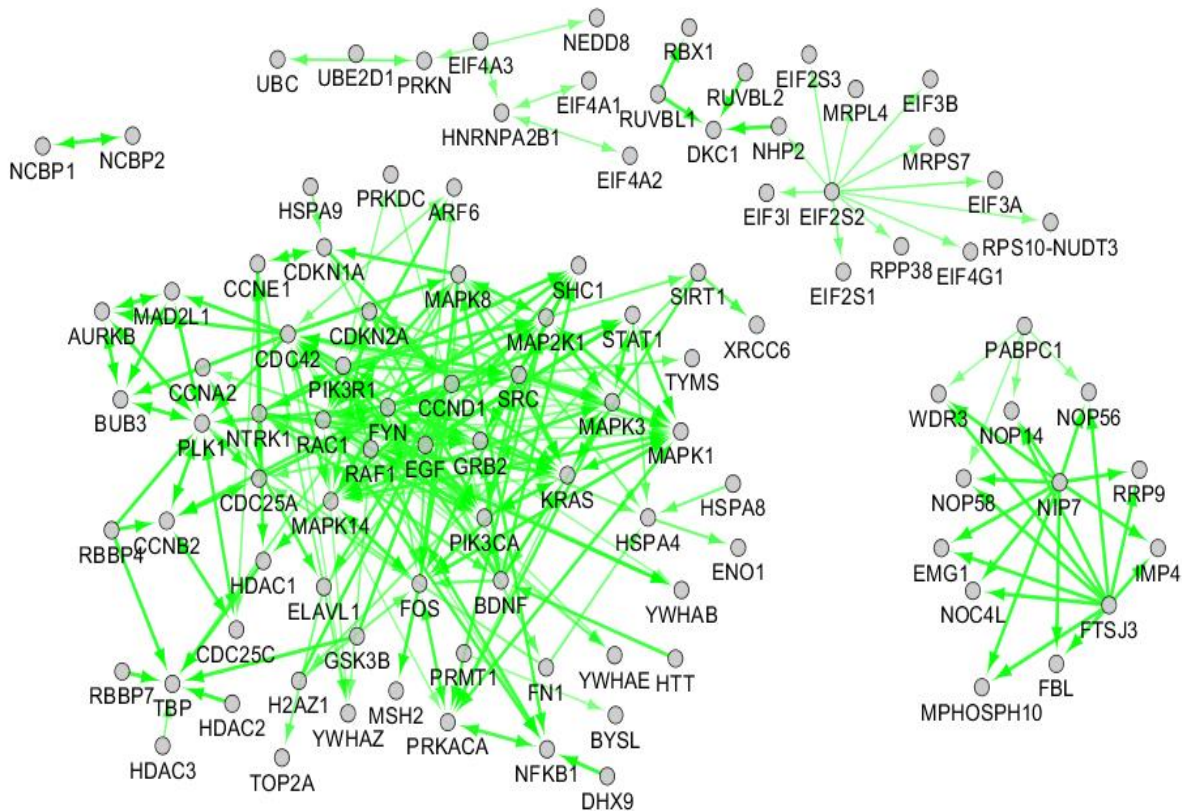


Figure 6. Directed PPI network of the hubs of the undirected PPI network including activation connections.

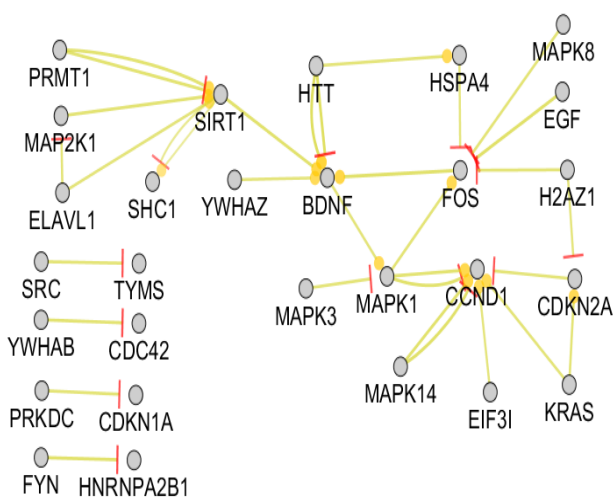


Figure 7. Directed PPI network of the hubs of the undirected PPI network including expression links.

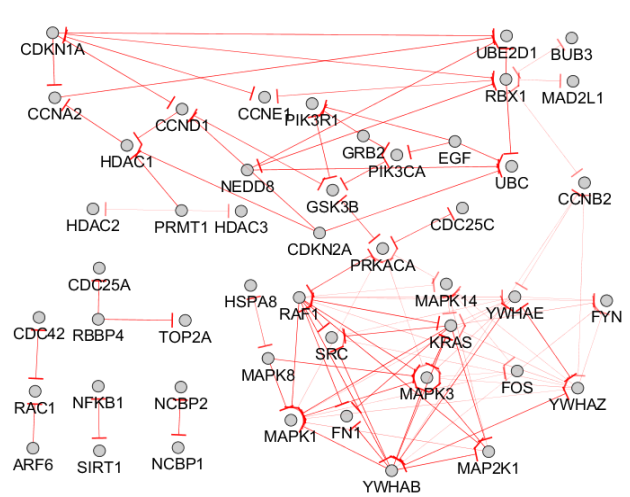


Figure 8. Directed PPI network of the hubs of the undirected PPI network including inhibition edges

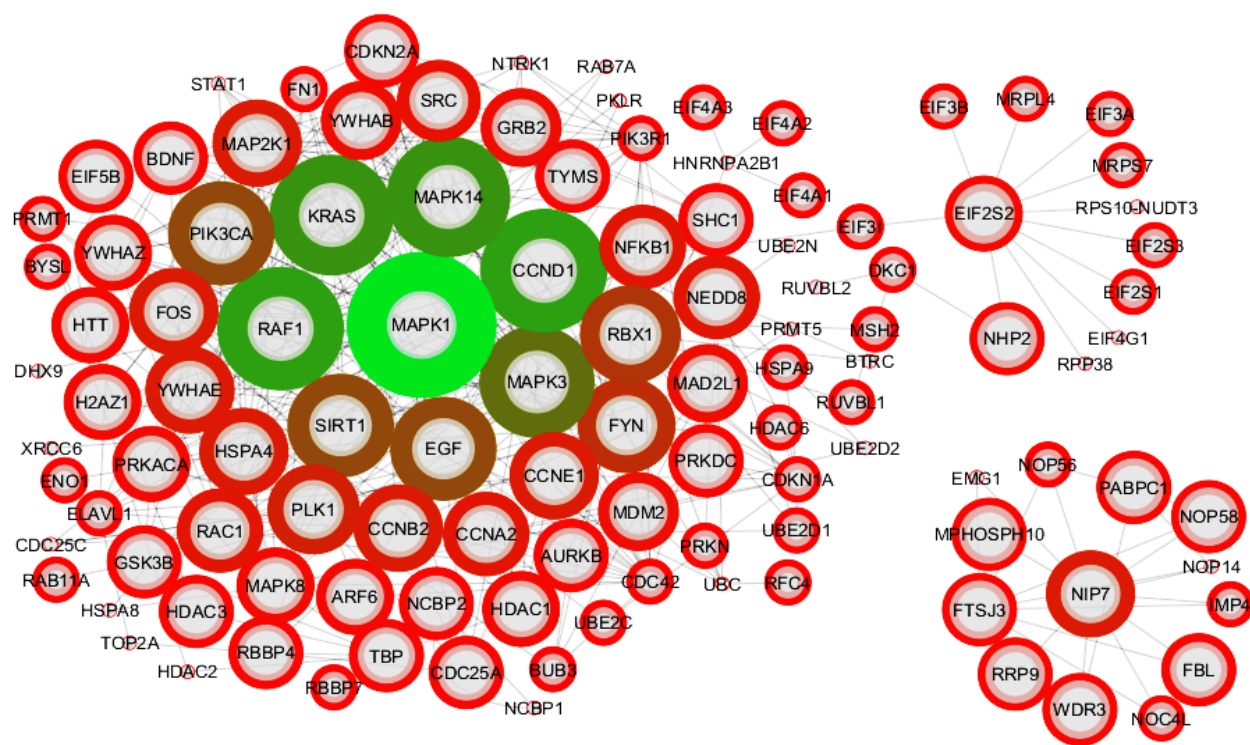


Figure 9. The directed PPI network including a subnetwork of 13 genes, and a main connected component of 98 nodes was analyzed and arranged based on out-degree value. The bigger size of nodes and red to green correspond to larger amounts of out-degree values.

Table 1. The up and down-regulated genes of human HepG2 cells in the presence of nisin are extracted from the document of Zainodini N et al [10].

No.	Gene symbol	Fold change	No.	Gene symbol	Fold change	No.	Gene symbol	Fold change
1	APAF1	-2.51	27	CD40LG	1.22	53	TRADD	-1.14
2	BAD	1.45	28	CD70	1.62	54	BAK1	2.47
3	BID	2.66	29	CFLAR	-2.99	55	BAX	-1.83
4	BIK	-4.89	30	CIDEA	-1.28	56	BAG1	2.99
5	BCL10	-1.58	31	CIDEB	-1.36	57	BAG3	3.28
6	BCL2L11	-1.22	32	CRADD	-3.61	58	BAG4	2.36
7	BCLAF1	2.23	33	DAPK1	-10.38	59	BCL2	-2.59
8	BNIP2	1.17	34	DFFA	1.19	60	BCL2A1	5.76
9	BNIP3	-1.13	35	FADD	2.49	61	BCL2L2	1.48
10	BNIP3L	2.34	36	FAS	-1.14	62	BCL2L10	-2.8
11	BRAF	-4.26	37	FASLG	-1.14	63	BFAR	-1.09
12	CARD6	3.27	38	HRK	3.42	64	BIRC3	-3.56
13	CARD8	1.79	39	LTA	-1.53	65	BIRC6	-1.97
14	CASP1	10.94	40	LTBR	3.26	66	BIRC8	-1.21
15	CASP2	-1.51	41	NOD1	-2.25	67	BNIP1	1.11
16	CASP3	-2.03	41	PYCARD	-1.64	68	IGF1R	-1.17
17	CASP4	1.24	43	TNF	6.62	69	MCL1	1.95
18	CASP5	2.29	44	TNFSF8	-1.14	70	NAIP	-2.6
19	CASP6	9.72	45	TNFSF10	1.14	71	NOL3	-1.57
20	CASP7	28.83	46	TNFRSF1A	10.27	72	RIPK2	-1.23
21	CASP8	-1.46	47	TNFRSF10A	-1.14	73	TP53	-1.74
22	CASP9	70.7	48	TNFRSF10B	-2.53	74	TP73	-4.15
23	CASP10	5.11	49	TNFRSF9	1.55	75	TRAF2	-2.21
24	CASP14	1.56	50	TNFRSF11B	-1.14	76	TRAF3	-2.67
25	CD27	1.12	51	TNFRSF21	-1.14	77	TRAF4	-3.52
26	CD40	-1.3	52	TNFRSF25	-6.612	78	XIAP	1.3



One of the attractive tools in biomedical investigation is transcriptomic analysis. UMAP as a non-linear method can efficiently group assorted samples in single-cell RNA sequencing analysis [19]. The clustering of the treated samples with sodium benzoate from the control cells is depicted in Figure 1. This finding corresponds to differences between gene expression profiles of the treated cells and control individuals. The other visualization method is a volcano plot which shows unstandardized signals such as log-fold-change versus noise-adjusted/standardized signals as like $-\log_{10}(\text{p-value})$ from the t-test. Volcano plot provides a double filtering tool to display screened data [20]. Volcano plot presentation of data in Figure 2 displays a large number of significant up and downregulated DEGs (11521 DEGs (see Figure 3) in response to the presence of sodium benzoate in cell culture media.

PPI network analysis is an attractive tool to assess large numbers of genes or proteins to explore critical individuals. For instance, the down-regulation of ALB and upregulation of JUN, FOS, and MYC as critical molecular events in response to okadaic acid by human intestinal caco-2 cells is detected via PPI network analysis [21]. In the present study, 208 hub DEGs were identified as the central genes associated to the effect of sodium benzoate on the treated cells. Gene co-expression networks are a widely useful approach to detect the system-level functionality of genes. The nodes in gene co-expression networks are connected via significantly co-expressed relationships between them. Gene activation and inhibition are highlighted to interpret gene regulatory networks [22, 23].

Activation, inhibition, and expression relationship between the introduced hubs are shown in Figure 4. Complex features of the action map (see Figure 4), required the presentation of each action in a related simple map (see Figures 5-8). As depicted in Figures 4 and 5, activation is a prominent action between the nodes of the network and NIP7 appears as a regulatory (activator) gene. Activation connections between nodes of network are so complex (see Figure 6) and understanding of relationships between the studied DEGs requires precious analysis. As shown in Figure 7, expression action appeared as the simplest network and included a minimum number of nodes.

One of the well-organized neighborhood-based metrics is degree value which has been extensively used due to its simplicity and low computation complication. The directionality of edges is not considered between the nodes, this problem limits the usage of degree centrality in a directed network. In a directed network, the kind of relationship between the nodes has significant importance. Therefore out-degree and in-degree values are appeared as the major tools to analyze the nodes of network [24]. The directed network of hub nodes (see Figure 4) was analyzed and the network was arranged based on out-degree value.

The result is shown in Figure 9. As depicted in Figure 9, MAPK1, CCND1, MAPK14, RAF1, KRAS, MAPK3, PIK3CA, SIRT1, EGF, RBX1, FYN, and NIP7 were pointed out as the crucial DEGs. As shown in Figure 6, all crucial genes (100%) are presented in the 3 significant subnetworks of the activation map while MAPK1, CCND1, MAPK14, KRAS, MAPK3, SIRT1, and EGF (58%) are included in the main connected component of expression map (see Figure 7). As shown in Figure 8, except for SIRT1 and NIP7 other crucial DEGs (83%) are included in the inhibition map. It can be concluded that activation, inhibition, and expression are respectively the main actions between the nodes.

Nucleolar pre-rRNA processing protein NIP7 is a downregulated hub gene that is highlighted in the activation map. Investigation indicates that downregulation of NIP7 is associated with pre-rRNA processing, leading to an inequality of the 40S/60S subunit ratio and reduction of the 34S pre-rRNA concentration and an intensification of the 26S and 21S pre-rRNA concentrations. This finding corresponds with cell proliferation alteration in human cells [25]. MAPK1, MAPK3, and MAPK14 belong to mitogen-activated protein kinase (MAPK) gene family, which are involved in regulation of cytokines and proteases expression, cell adherence, cell cycle progression, cell motility and metabolism. The effect of MAPKs on cell proliferation, survival, differentiation, development, and apoptosis is investigated and reported [26, 27]. There is evidence that the downregulation of Raf-1 proto-oncogene, serine/threonine kinase (RAF1) and upregulation of cyclin D1 (CCND1) is accompanied by HepG2 cells proliferation and migration induction [28]. The downregulation of RAF1 and the upregulation of CCND1 in response to the presence of sodium benzoate in human HepG2 cells are pointed out in the present study.

Investigation indicates that overexpression of miR-30c prevents proliferation, migration and invasion of prostate cancer cell lines. This effect of miR-30c is demonstrated via the downregulation of KRAS proto-oncogene, GTPase (KRAS) protein by miR-30c [29]. KRAS is downregulated in the presence of sodium benzoate. EGF is another upregulated gene in response to sodium benzoate effect on the treated cells. There is evidence that EGF induces tumor cell invasion [30]. Sirtuin-1 (SIRT1) is a downregulated hub, this gene is known as a protective element against the progression and development of non-alcoholic fatty liver disease [31]. It is reported that phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) upregulation is associated with several tumors [32]. PIK3CA has been upregulated in the presence of sodium benzoate. Investigation revealed a significant role of ring-box 1 (RBX1) in the development of non-small cell lung cancer [33]. It appeared as a downregulated hub in the



present study. FYN proto-oncogene, Src family tyrosine kinase (FYN) is the last downregulated gene, upregulation of FYN in thyroid carcinoma is reported by Zheng et al [34].

The apoptosis-related dysregulated genes in response to the presence of nisin are shown in Table 1. The assessment showed except for TNRSF25 there are no common genes between these dysregulated genes and the significant DEGs of sodium benzoate analysis. TNRSF25 is upregulated in the presence of sodium benzoate 2-fold while is downregulated in the presence of nisin 6.6-fold. Both up and down-regulation of TNRSF25 refer to the side effects of sodium benzoate and nisin effect as food preservative agents. Findings indicate that nisin activity against apoptosis is different from sodium benzoate. It seems nisin has a potent effect on cellular apoptosis relative to sodium benzoate.

4. Conclusion

In conclusion, the basic functional features of human HepG2 cells are affected by sodium benzoate. Discrimination of the 40S/60S subunit ratio, decrease of the 34S pre-rRNA concentration, intensification of the 26S and 21S pre-rRNA concentrations, regulation of cytokines, some cancerous processes expression, cell adherence, cell cycle progression, cell motility and metabolism, cell proliferation, survival, differentiation, development, and apoptosis, tumor cell invasion, and progression and development of non-alcoholic fatty liver disease related pathways are the highlighted targets of sodium benzoate. The mentioned alterations include positive and negative functional changes. However, the negative side effects are dominant relative to the benefits. Nisin analysis showed that many apoptotic-related genes were dysregulated in response to the presence of nisin. However, this effect was not similar to the response of cells to the presence of sodium benzoate. Based on findings, chemical and bio-preservative food additives can affect human cellular function in various manners which influence human health. Finally, it is difficult to introduce nisin as a safer food preservative compound relative to sodium benzoate.

5. Acknowledgements

Shahid Beheshti University of Medical Sciences supported this research.

6. Conflict of Interest

The authors report no conflicts of interest.

7. Author contributions

Mostafa Rezaei-Tavirani is contributed to the conception and design of the work and literature review. All authors participated equally in project administration and writing of the first draft of manuscripts, providing critical revision and

editing. All authors approved the final version of the manuscript.

8. Ethical Code

This project is approved via [IR.SBMU.RETECH.REC.1403.105](https://doi.org/10.3390/nu14071497) Ethical code.

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اثر بنزوات سدیم و نایسین بر بیان ژنی سلول های HepG2 انسانی

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چکیده

سابقه و هدف: بنزوات سدیم به دلیل خواص ضد باکتریایی و ضد قارچی که دارد، به عنوان یک ماده نگهدارنده واجد ایمنی بالا در صنایع غذایی و دارویی شناخته می شود. از طرفی، نایسین یک ماده نگهدارنده زیستی است. هدف از این مطالعه تعیین اثر بنزوات سدیم بر بیان ژنی سلول های HepG2 در مقایسه با نایسین است.

مواد و روش ها: اثر بنزوات سدیم بر بیان ژنی سلول های HepG2 انسانی از پایگاه داده Gene Expression Omnibus (GEO) استخراج شد. تجزیه و تحلیل قبل از ارزیابی از طریق برنامه GEO2R انجام شد. ژن هایی که بصورت افتراقی بیان شده بودند (DEGs) از طریق آنالیز شبکه تعامل پروتئین-پروتئین (PPI) مورد بررسی قرار گرفتند. هاب های شبکه مورد بحث از طریق یک شبکه جهت دار غریبال شدند. ژنهای هاب مهم شناسایی و مورد بحث قرار گرفتند. ژن های مرتبط با آپوپتوز سلول های HepG2 انسانی تعیین و از طریق تحقیقات انجام شده در گذشته بررسی قرار گرفتند.

یافته ها و نتیجه گیری: در مجموع ۱۱۵۲۱ ژن که بصورت افتراقی بیان شده بودند شناسایی شدند. سرانجام شبکه تعامل پروتئین-پروتئین شامل ۴۰۹۵ DEG و ۲۰۸ هاب ایجاد شد. هاب ها از طریق یک شبکه پروتئینی جهت دار ارزیابی شدند و ژن های EGF, SIRT1, PIK3CA, MAPK3, KRAS, RAF1, MAPK14, CCND1, MAPK1, NIP7 و FYN, RBX1 به عنوان ژن های هاب حیاتی در پاسخ به حضور بنزوات سدیم معرفی شدند. از مجموع ۷۸ ژن مرتبط با اثر نایسین بر سلول های مورد بحث (به جز TNRSF25) هیچ ژن مشترکی با DEG های ناشی از ارزیابی اثر بنزوات سدیم نداشتند. می توان چنین نتیجه گرفت که بنزوات سدیم و نایسین به طرق مختلف بر عملکردهای ضروری سلولی مانند پیشرفت چرخه سلولی، تحرک و متابولیسم سلولی، تکثیر سلولی و بقا تأثیر می گذارند. به نظر می رسد استفاده گسترده از عوامل نگهدارنده مواد غذایی نیازمند بررسی بیشتر برای تضمین حفظ سلامت انسان است.

تعارض منافع: نویسندگان اعلام می کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.