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RESEARCH PAPER

BIOTECH STUDIES

Development of doubled haploid maize lines by using *in vivo* haploid technique

Rahime Cengiz[1]*, Kayıhan Zahit Korkut[2]

[1]Sakarya University of Applied Sciences, Field Crops Department, Sakarya, Turkey.
[2]Tekirdağ Namık Kemal University, Field Crops Department, Tekirdağ, Turkey.

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***Corresponding Author**
Tel.: +90 555 967 0353
E-mail: rcengiz24@gmail.com

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Abstract

The doubled haploid technology is now an integral component of modern maize breeding programs. In this study, the maternal haploid induction (gynogenesis) method was used to derive Doubled-Haploid (DH) lines from elite maize germplasm adapted to Turkey. Temperate haploid inducers (RWS, RWK-76, RWS x RWK-76 and WS14) were used as pollinators, and a set of 30 single-crossses (in FAO 650-700 maturity groups) were used as source materials. Putative haploid seeds were selected based on expression of *R1-nj* anthocyanin color marker. Highest haploid induction rate (20.42%) was recorded by using RWK-76 as inducer line, and the lowest haploid induction rate (17.75%) was obtained through WS14. Putative haploid seeds were germinated and seedlings were treated with 0.06% colchicine + 0.5% dimethylsulfoxide solution. Following transfer of seedlings into the field, 2178 D_0 plants were obtained out of a total of 3012 treated haploids. Live plants were from 89% of 2178 seedlings which are planted to the field. Fertile plants were formed 57% of live plants. Inbreeding was succeeded in 31.23% of fertile plants and only 7.8% of inbreeding plants were able to produce seeds. Consequently, 27 doubled haploid lines were developed.

Introduction

Obtaining of inbred lines is the first and basic step in hybrid maize breeding. The aim of self-pollination is to obtain homozygous lines to be used as parents in developing the hybrid variety. At least seven generations of selfing is required to obtain an inbred line with 99% of homozygosity. Obtaining inbred lines is the most time-consuming part of the hybrid maize variety development process. New alternative complementary and supporting methods can be used to shorten the conventional hybrid maize breeding program (Cengiz et al., 2013).

Chase (1951) demonstrated the practical application of DH in maize breeding. *In vivo* haploid induction is now widely used in maize research and breeding. DH mapping populations are used to analyze linkage disequilibrium and analysis of haplotype-trait associations. In breeding, DH lines have enabled increased efficiency of line development and recurrent selection, and to reduce the effort for line maintenance (Röber et al., 2005).

The researchers stated that there are known two methods of *in vivo* haploid technique. Both are paternal haploid method which is used in order to obtain cytoplasmic male sterile form of elite line. Inducer line used in this technique carries the mutant indeterminate gametophyte recessive gene as female, unlike the inducer line used in the maternal haploid technique. Maternal haploid method is used for developing DH lines. Comparing the *in vivo* and *in vitro* haploid induction methods, the response of donor germplasm to the success of haploid achievement in the *in vitro* technique is very effective, whereas this success in the *in vivo* technique is less dependent on the donor

response (Geiger 2009; Spitkó et al., 2006; Röber et al., 2005).

Geiger (2009) indicated that the most efficient haploid identification marker is the 'red crown' or 'navajo' kernel trait encoded by the dominant mutant allele *R1-nj* of the 'red color' gene *R1*. There are different dominant genes such as *A1*, *A2* and *C2* in the maize plant for coloration. The dominant gene *R1-nj* reveals deep coloration in the embryo and endosperm.

Using *in vivo* maternal haploid method, inducer lines are crossed to the donor germplasm not having the anthocyanin color markers at kernel, all the resulting hybrid kernels are expected to express the red color phenotype in the endosperm and in the scutellum as *R1-nj* is dominant over the colorless *r1* allele. Thus, the differential expression of *R1-nj* facilitates identification of maternal haploids from the diploid kernels. When maternal inducer lines with a high haploid induction rate (HIR) are used in the induction cross, putative haploids usually happen at a frequency of 6-10% (Chaikam & Prasanna, 2012).

Gallais (1990) examined the effect of additive variance of germplasm to be used in obtaining DH lines. In this study, the covariance between the lines was calculated by evaluating the line value together with the additive variance. As a result, it was estimated that a DH line derived from the F_2 population combined about 50% better alleles than those derived from the F_1 population.

Prigge et al. (2011) have used hybrids, landraces and open-pollinated varieties as the source material for *in vivo* maternal haploid induction. Also, HIRs were compared between the dent and flint types. A significant variation was observed in terms of haploid induction in different source materials. Single-cross hybrids were found to have higher HIR than other source materials.

Hybrid maize breeding programs in Turkey have recently started using *in vivo* maternal haploid technique. This is the first study to report development of maternal haploids in Turkish maize germplasm using *in vivo* haploid induction. The aim of the study was to effectively use temperate haploid inducers in induction crosses hybrid maize varieties developed by Maize Research Institute, Sakarya, Turkey, and further develop DH lines using chromosome doubling of maternal haploids.

Materials and Methods

Materials

This study was conducted at Maize Research Institute, Sakarya, Turkey. A set of 30 elite single-cross hybrids (FAO 650-700 maturity group), were used as source materials. Of these 30 single-cross hybrids, 25 have dent kernel type and five had flint-like kernels.

The temperate haploid inducer lines RWS and RWK-76 and their cross RWS x RWK-76 were sourced from the University of Hohenheim, Germany, while the inducer line WS14 was obtained from French National Institute for Agricultural Research (INRA). The inducer genotypes used in this study had *R1-nj* allele (Figure 1) with anthocyanin color expression on the crown region of the endosperm and the embryo.

Methods

Inducer line was used as pollinators in *in vivo* maternal haploid technique. The source material was main donor and used as female parent. The source material used 30 single-cross hybrids were planted 3 rows. Tassels of source materials were removed by cutting on flowering period. Ears of source materials and tassels of inducer lines (50% anthesis) were covered with papers. Every row of hybrid varieties was hybridized with each inducer line. Fertilized ears were kept in isolation papers until harvest. The names of the pollinators were written on isolation paper.

HIR was determined as below:

HIR = (Number of haploids/Total number of seeds) x 100

In vivo maternal haploid technique includes the following steps:

(I) Crossing the donors (e.g. individuals belonging to F_1, F_2 populations) as female parent with the inducer lines as male parent.
(II) Selection of putative haploid kernels (at the mature seed or root of seedlings) using the anthocyanin color markers.
(III) Germination of the putative haploid seeds.
(IV) Determination of effective and nature-friendly chromosome doubling agent and treatment to the putative haploid seedlings.
(V) Growing seedlings in optimum conditions and practice to necessary agronomic management

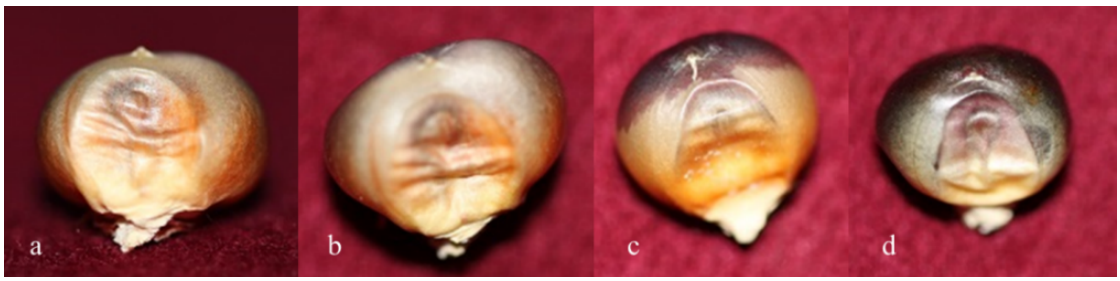

Figure 1. Expression of R1-nj color marker of inducer lines, (a) RWSxRWK-76, (b) RWK-76, (c) RWS, (d) WS14.

of D_0 seedlings and obtain of DH seed by self-fertilization D_0 plants.

(VI) Recurrent selection and application of DH lines in breeding programs.

Haploid kernels were identified using the anthocyanin color marker in the seeds obtained from induction cross (Figure 2).

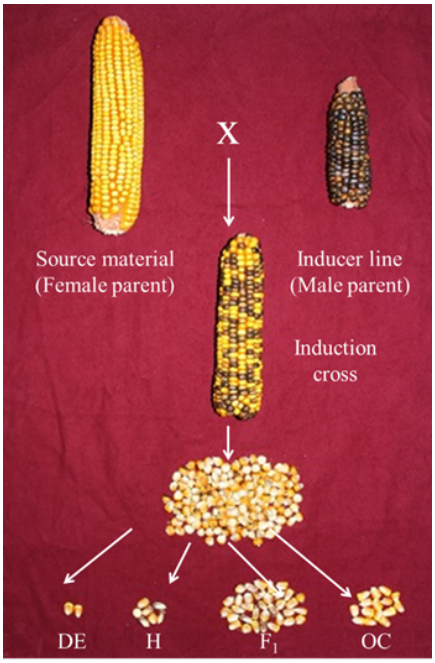

Figure 2. Haploid seed identification based on *R1-nj* marker expression. OC; outcross seeds, F_1; hybrid seeds with inducer line, H; putative haploid seeds, DE; seeds with diploid endosperm.

Haploid seeds were germinated and treated the chromosome doubling agent at the coleoptile stage, 3 days after germination. Chromosome doubling protocol was followed based on Deimling et al. (1997). The whole seedlings were immersed into a 0.06% colchicine solution plus 0.5% dimethyl sulfoxide (DMSO) for 12 h at 20°C. After the colchicine treatment, the seedlings were carefully washed in water and planted and grown in pots (during the first days under high humidity) in the growth chamber upto 3-4 leaf stage. A few weeks later the plants were transferred to field. Self-pollination of the D_0 plants were done.

Results and Discussion

Productivity pollen ability of RWS and RWK-76 lines are less than normal maize plants. Therefore, the number of seeds rate from obtained induction cross were low as against RWSxRWK-76. The number of pollinator plants of WS14 were high for producing seeds although ability of pollen production of WS14 was also low. A high number of plants increased seed formation rate as a result of induction cross.

Four different types of seeds were obtained from induction hybridization. In normal diploid or hybrid seeds, the endosperm and embryo had a purple color. Although the putative haploid seeds and their endosperm had purple color, embryo of these seeds was colorless. On the other hand, in the seeds with diploid endosperm, the endosperm was colorless, while embryo was colorful. In putative outcross seeds, the embryo and endosperm were not colorful. The seeds obtained from the induction cross were divided according to these criterias given in Figure 3.

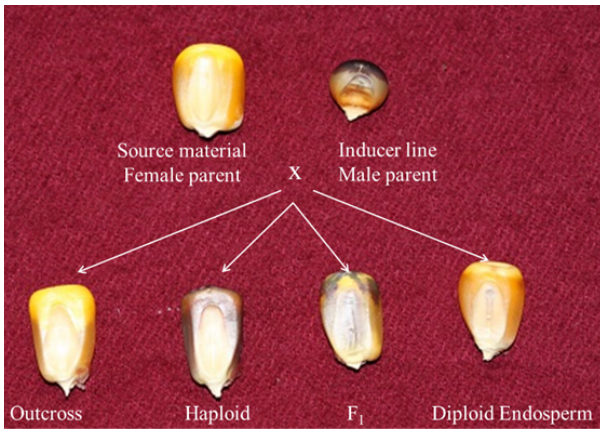

Figure 3. Identification of haploid seed based on R1-nj color marker.

The seeds obtained from induction hybridization with each inducer genotype were made distinction based on the four categories. Number of haploid seeds and the other categories on seeds obtained from induction hybridization were given on Table 1. HIR and rates of the other categories were given on Table 2. The highest haploid induction rate is determined in RWK-76 inducer line although HIR of inducer genotypes were varied. According to the average of the studies carried out with many donors in different environments, RWS has an induction rate of about 8%, RWK-76 recorded an average induction rate of 9-10%, and the same rate was observed for the cross RWS x RWK-76. WS14 had about 2-5% haploid induction rate (Geiger 2009; Lashermes & Beckert 1988). Most seeds belonged to F_1 category (54.14-68.01%) and rate of seed with diploid endosperm was very low.

Chaikam and Prasanna (2012) indicated that *R1-nj* marker system offers an efficient way to determine haploids, but *R1-nj* expression could be highly influenced by the genetic background of the donor parent. The Navajo crown coloration might vary from a small spot (at the silk attachment point of the seed) to covering the entire aleurone (except the base). In addition, the intensity of color on the aleurone and scutellum may vary from very pale to deep. We found similar results to the study of these researchers (Figure 4). Differences in the occurrence of *R1-nj* may lead to misgrading. It is impossible to identify haploids when it is completely inhibited on both endosperm and embryo. All source germplasm in this study showed *R1-nj* expression on both endosperm and embryo. So, these dent and flint-like donor hybrids do not include inhibitor gene.

Table.1. Number of haploid and the other categories of seeds as total number obtained from induction hybridization.

Inducer genotypes	Seed number			
	Haploids	Diploid endosperm	F_1	Outcross
RWS x RWK-76	922	19	3215	571
RWS	410	11	1256	392
RWK-76	473	15	1403	425
WS14	1207	25	3681	1886
Total	3012	70	9555	3274
Grand total		15911		

Table.2. HIR and the rates of the other categories calculated using total numbers of seeds obtained from induction hybridization.

Inducer genotypes	%			
	HIR	Diploid endosperm	F_1	Outcross
RWS x RWK-76	19.50	0.40	68.01	12.08
RWS	19.82	0.53	60.71	18.95
RWK-76	20.42	0.65	60.58	18.35
WS14	17.75	0.37	54.14	27.74
Average of HIR	18.93			

Some seeds were also lack of endosperm and embryo kernel formation as a result of haploid induction (Figure 5). Those seeds did not have germination ability and these numbers of seeds were not included in the other categories.

The precision and speed of putative haploid seed selection depend on the qualification of employees with good understanding of haploid identification through the *R1-nj* expression on aleurone and scutellum. According to the experiences, a light-flooded working environment and doing no more selection than four hours are critical.

Haploid identification is a lengthy process when there are a large number of seeds. Haploid identification can get difficult due to the limitations of *R1-nj* system. *B1* and *Pl1* system have also got limitation such as some germplasms also have purple root features (Rotarenco et al., 2010). Some researchers used original marker systems that could potentially facilitate automated haploid identification with the least false positives by using kernel oil content (Melchinger et al., 2014; Rotarenco et al., 2007). However, the source germplasm includes low oil content. Therefore, an automation system should be developed for the identification of different categories of seeds. These kinds of systems, like scanning the seeds as visually and by their content, would facilitate the identification of the haploid.

Altuntaş et al. (2018) used 87 haploid and 326 diploid maize seeds as their dataset. They used the expression of the *R1-nj* color marker in the embryo and endosperm. These features were obtained from gray

Figure 4. Showing distinctness in *R1-nj* expression on the aleurone and scutellum.

Figure 5. The seeds obtained from induction hybridization that were not included to the other categories, **(a)** without endosperm, **(b)** without embryo.

level co-occurrence matrix. The feature vectors were classified using decision trees, k-nearest neighbors and artificial neural networks. The classification performance of machine learning tecniques was tested by using a 10-fold cross-validation method. As a result of this test, the best performance was measured in decision tree with the classification success rate as 84.48%.

Seeds with a haploid embryo include a regular triploid (3n) endosperm, for this reason, these seeds are adequate to show germination similar to those seeds with a diploid embryo (Coe & Sarkar 1964). Haploid seeds were germinated in dark growth chamber at 23°C for 3 days. Putative haploid seedlings with a root length of 3 cm and coleoptile length of about 1.5-2 cm are ideal for colchicine application. Before colchicine application, root and coleoptile tissues were cut at about 2 cm and 1 cm from the tip, respectively, using a sterile scalpel blade or clippers. Cut seedlings that belong to the same single cross were kept in a mesh bag with its tag. Mesh bags with seedlings were kept in water till transferring to the colchicine tank.

Colchicine is the agent most commonly used to induce chromosome doubling. Colchicine disrupts mitosis by binding to tubulin, thus inhibiting the formation of microtubules and the polar migration of chromosomes, resulting in a cell with a doubled chromosome number (Barnabas et al., 1999). Chromosome doubling was applied in accordance with Deimling et al. (1997). After the colchicine application, the seedlings were planted in pots afterwards grown (during the 3 days under high humidity) in the growth chamber to the 3 to 4 leaf stage. D_0 seedlings were transplanted into the field manually. D_0 seedlings need less water than normal maize since they have weaker roots. For this reason, it is vital that the correct quantity of water is treated for good plant development. Maintenance of seedlings in the field was properly made such as fertilization application, weed control as manually and insect control. When D_0 plants came into flowering stage, self-pollinating was made manually on D_0 plants and DH seeds were taken at harvest.

We observed that, after application of colchicine, all chromosomes of each cell in a seedling might not duplicate properly called sectoral diploidization. Some D_0 plants had tassels showing good production of pollen. Mostly D_0 plants had tassels producing small amounts of pollen or had anthers without pollen (Figure 6). Therefore, self-fertilization was forced to be done as repetitively.

DH ears were harvested by waiting for appropriate harvest moisture. Ears with red-colored seeds were discarded because they resulted from misclassified seed that could have been missed in the earlier steps. Frequently few seeds were set on the ears of DH plants (Figure 7).

Accordingly, we took utmost care to avoid seed loss in the field. The ears were carefully harvested by workers and kept in appropriate paper bags during transport to the storage for dehusking and drying. The seed representing the novel developed, entirely homozygous DH line will be sowed again for seed reproduction to be able to use the DH line in the future studies and breeding actions. The amount of seeds obtained from the first generation of DH lines are given in Figure 8.

Conclusion

In this study, 2178 D_0 plants were obtained out of a total of 3012 treated haploids. The viability rate of 2178 seedlings transferred to the field was 89%, and 57% of these surviving plants were fertile. Inbreeding was succeeded in 31.23% of fertile plants and only 7.8% of inbreeding plants were able to produce seeds. Consequently, 27 DH lines were developed.

Figure 6. Tassel with a few branches are producing pollen which were sterile due to sectoral diploidization (left) and a completely sterile tassel (right).

Figure 7. Some harvested ears of D_0.

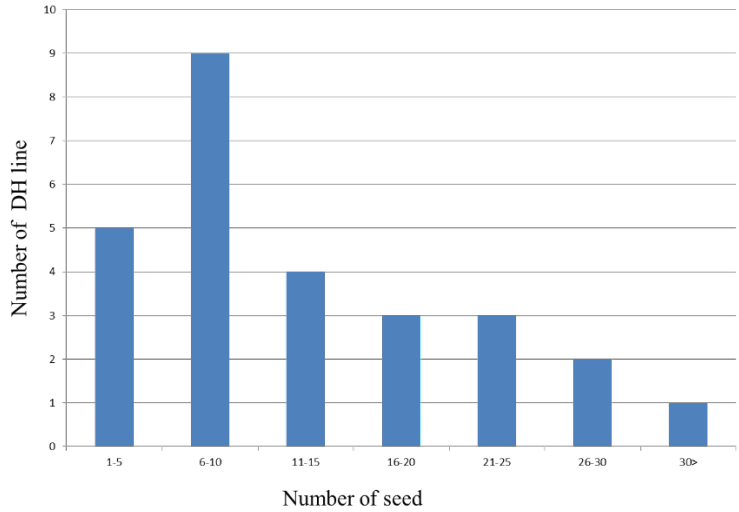

Figure 8. Amount of the seeds obtained from the first generation of DH lines.

Improvement of DH lines by *in vivo* maternal haploid induction needs specific abilities and equipments for i) great-quantity of chromosome doubling, ii) growing of putative haploid seedlings in the greenhouse, iii) transferring the seedlings to the field, iv) application of good growing conditions to seedlings, v) self-pollination of the D_0 plants.

In vivo maternal haploid technique has been an indispensable method of advanced breeding programs worldwide. Public research institutes and some private sector of carrying out maize breeding program in Turkey have started application *in vivo* maternal haploid technique in recent years.

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RESEARCH PAPER

Meta-analysis of transcriptome data on oxidative stress response in *Saccharomyces cerevisiae* cells underlines regulation of carbon, redox and glutathione metabolism

Ezgi Tanıl, Burcu Şirin, Emrah Nikerel*

Yeditepe University, Faculty of Engineering, Department of Genetics and Bioengineering, 34755 Ataşehir, Istanbul, Turkey.

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***Corresponding Author**
Tel: +90 (216) 578 0619
E-mail:
emrah.nikerel@yeditepe.edu.tr

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Abstract

Environmental stress adversely affects living systems within medical as well as industrial context, causing either diseases or resulting in e.g. underperforming production processes. In particular oxidative stress in industrial biotechnology context, manifested as the imbalance in generation of reactive oxygen species and antioxidant capacity causes yield losses both in growth and production in baker's yeast. Oxidative stress response studies for *Saccharomyces cerevisiae* at transcriptome level are using either direct induction methods such as treatment with peroxides or indirect induction methods such as treatment with drugs or toxins. To extract common response mechanisms integrating all conditions is of high value. To this end, this study collects, processes and aggregates published transcriptome data from studies that examined the response using both direct and indirect oxidative stress induction methods. Interestingly, carbon metabolism, oxidation reduction processes and glutathione metabolic process were found to be the common mechanisms involved in oxidative stress response. However, ion homeostasis and hexose transport mechanisms have been shown to be affected from direct induction using peroxides. This result illustrates bioinformatics analysis for large, aggregated transcriptome datasets, as a steppingstone for finding common features and further metabolic engineering targets were developed.

Introduction

Oxidative stress is ubiquitous in living systems from microorganisms to plants and mammalian systems, manifested as the imbalance between the generation of reactive oxygen species (ROS) and the capacity of the biological system to detoxify these intermediates (Inzé & Van Montagu, 1995; Sies, 2000; Storz & Imlayt, 1999). The excess free radicals result in damage to fatty tissue, DNA, and proteins causing in turn loss of function, yield or life quality. Coupled with redox imbalance, oxidative stress typically induces system-wide response, involving several transcripts, proteins and biochemical reactions, spanning several omic-layers.

Cells develop complex responses and protection mechanisms to maintain intracellular redox balances against oxidative stress. Responses to oxidative stress are usually on cell survival or cell death mechanisms. Cell survival mechanisms involves antioxidant enzyme mechanisms (SOD, catalase, peroxidase reactions), metabolic products (glutathione, used in almost all eukaryotes) (Poljak et al., 2003), upregulation of various transcription factors (Yap1p, Skn7p, Msn2/4p (Diezmann, 2014)). Besides, necrosis, apoptosis and autophagy are the cell death mechanisms against oxidative stress. These programmed cell death responses of the cell shed light on neurodegenerative diseases and cancer studies (Figure 1) (Farrugia & Balzan, 2012; Harding et al., 2003; Luo et al., 2017).

Bakers yeast, *Saccharomyces cerevisiae*, has long been used for centuries not only for making bread or various drinks, but also as food supplement, feed additive (Newbold et al., 1996) as well as for production of bioplastics and other biomaterials (Breuer et al., 2002), biofuels (Cardona et al., 2009) and within medical context (Borresen et al., 2012). As such, it is considered

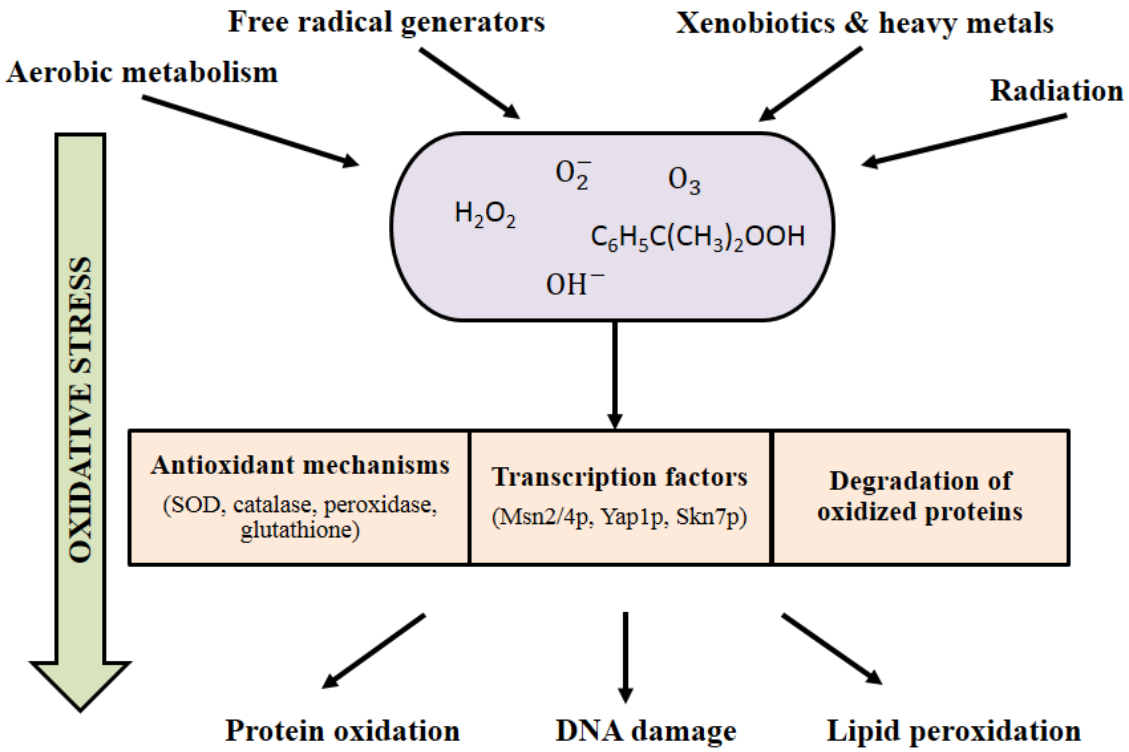

Figure1. A general overview for causes and possible consequences of oxidative stress.

as a model organism for eukaryotic systems as well as an important workhorse for industrial biotechnology (Giaever et al., 2002; Hartwell, 1974; Lee et al., 2002; Li et al., 2004; Mager & Winderickx, 2005; Nevoigt, 2008; Oliver, 1996; Petranovic & Vemuri, 2009). The availability of genome sequence (Wei et al., 2007) as well as various molecular biology tools (McIsaac et al., 2014) renders yeast central not only to understand how cellular machinery works but also for further metabolic engineering studies.

Yeasts are exposed to various stresses (heat, pH, nutrient limitation, osmotic, oxidative) in the industrial context and/or changing environmental conditions. Oxidative stress is an inevitable part of the aerobic life cycles of yeasts. The oxidative stress response in yeast has been evaluated with different perspectives of finding toxicity level of stress agents (Flattery-O'Brien et al., 1993; Jamieson, 1992), identifying the cellular mechanisms (Davies et al., 1995; Gasch et al., 2000), determining stress related genes with mutant strains (Grant et al., 1996, 1998; Okada et al., 2014) and understanding the main mechanisms (Cyrne et al., 2003; Godon et al., 1998; Gopalacharyulu et al., 2009; Matallana & Aranda, 2017; Peláez-Soto et al., 2020; Piedrafita et al., 2015; Ralser et al., 2007; Yoshimoto et al., 2019). Stress responses have also been studied for living systems for various taxa, ranging from bacteria (Christodoulou et al., 2018; Zhai et al., 2020) to plant cells (Sipari et al., 2020) and other higher eukaryotes (Yu et al., 2020).

In particular, there are several studies focusing on transcriptomic (TX) response. Many studies that investigate transcription factors such as Yap1, Skn7 and Msn2/4 that regulates oxidative stress response (Carmel-Harel et al., 2001; Livas et al., 2011; Ma & Liu, 2010; Ouyang et al., 2011; Sha et al., 2013), antioxidant defense mechanisms such as catalases, SOD, thioredoxins and other cellular responses such as cell death and apoptosis following oxidative stress can be found in the literature. However these data obtained from different analysis platforms, oxidative stress inducers and many other experimental and analysis techniques (Cheng et al., 2018; Farrugia et al., 2019; Rodriguez-Colman et al., 2010). Although these studies provide useful information, they cannot be compared to other results of experiments, yet aggregation is of great interest to distill common features of stress response in yeast cells.

A significant challenge in analyzing large scale – omic datasets compiled from different sources is to extract key information from experiments performed individually with various agenda, using different techniques or agents, by different labs and/or technicians. In particular for oxidative stress, different labs used various agents (H_2O_2, CHP) to induce the stress, alternative techniques (cDNA microarray, oligonucleotide microarray, qPCR, RNA-Seq), alternative experimental design (time-course, static, comparison with a selected mutant) and various strains of *Saccharomyces cereviasiae* under different growth conditions. Even the purpose of each experiments ranges from determination of toxic/lethal effect to finding resistance genes to stress. A promising approach to analyze omic-dataset from different backgrounds is to perform so-called, meta-analysis of such data. In its essence, meta-analysis aims to aggregate available data

or information and find key features underlying the system of interest. The approach has been used within various context including oncogenetics (Bhasin et al., 2016), biomarker for plant biotechnology (Zimmermann et al., 2008), stem cell research (Assou et al., 2007) finding stress responsive pathways (Panahi et al., 2019) with even dedicated databases (Hruz et al., 2008).

Rank aggregation is one proposed method for meta-analysis e.g. to combine TX data obtained from different sources (Wald et al., 2012a, 2012b), since the results of high-throughput genomics experiments contain significant amount of noise, and thus reliability of the results should be supported by combining evidences from different experiments or platforms. Therefore, rank aggregation methods provide less noisy results by combining several preference lists. Different approaches proposed in the literature and Robust Rank Aggregation is one of the distribution based approaches which assumes a null probabilistic model to compare each ranked list (Li et al., 2019). The approach consists of focusing on the ranks of genes from individual transcriptome studies, rather than the expression value itself and aggregating list of ranks, assuming beta-distribution for the underlying population of lists and scores each element of the combined list with a corrected p-value. These corrected p-values are sorted to obtain final aggregated ranks. (Kolde et al., 2012; Li et al., 2019)

The advent of high-throughput omic technologies generated ever-growing immense amount of data, which in turn, remains largely under-used and under-interpreted. Making more of the available –omic data is the main approach of our work. The aim of this study is therefore to collect, analyze, aggregate and perform functional analysis of available transcriptome data from response of yeast cells to various oxidative stresses, within the scope of further understanding the effect of this stress on *Saccharomyces cerevisiae* cells and finding common features among different experiments using bioinformatics tools. In doing so, microarray datasets from NCBI database is collected, inspected for differential expression and compiled a large dataset containing differentially expressed genes in each experiment. Upon rank aggregation and downstream functional analysis, the implications within industrial biotechnology is discussed.

Materials and Methods

Data Acquisition and Differential Expression Analysis

All data used in this study has been compiled from public sources. Overall workflow to obtain the transcriptome dataset is presented in Figure 2, including the number of datasets in each step. NCBI Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/) was searched for the term “oxidative stress” and further filtered for “*Saccharomyces cerevisiae*”.

In this paper, we focused on microarray data, due to standardized experimental and analytical protocols and focused experimental context. In particular, the microarray results from the experiments measuring the cellular response against treatments with oxidants such as peroxides (e.g. H_2O_2, CHP), drugs (e.g. Epolauridine) and toxins (e.g. citrinin), directly causing oxidative stress. The list of datasets for the final meta-analysis is given in Table 1. For samples with replicates (23 of 34 samples), from documented commercial microarray platforms, Differentially Expressed Gene (DEG) analysis was performed using R limma package (two dye, dye swap and time course designs are also included) and the results were filtered for $p<0.1$ and log fold change, $logFC>1$. For the samples without replicates, the published logFC was used with the same criteria.

Table 1. Datasets used for meta-analysis.

Stress Inducer	Accession IDs
H_2O_2	GSE45370, GSE58992, GSE135546, GSE79037, GSE63030, GSE16346, GSE26829, GSE55081, GSE12220, GSE15936, GSE3406, GSE2977
Linoleic acid hydroperoxide (LoaOOH)	GSE18334, GSE47820, GSE54951,
CHP	GSE7645, GSE26169
Oxygen concentration	GSE22832
Transition from anaerobic to aerobic	GSE7140
TiO_2 + UV	GSE99660
Mycotoxins: citrinin (CIT) and ochratoxin A (OTA)	GSE84187
Pyocyanin	GSE6185
Eupolauridine-9591 (E9591)	GSE101749
Menadione	GSE3683
Celastrol	GSE5608
Arsenite	GSE6067, GSE6068
Zinc	GSE18411
Potassium	GSE24712

For time-course experiments (11 of 34), the last time point or, 30/60/90-minute time point (if available) was compared to control taken prior to treatment. For each sample, the absolute values of logFC upon filtering were ranked in increasing order and these ranks were normalized with respect to maximum rank in the corresponding sample. Finally, normalized ranks for each gene among all of the samples were merged to generate the rank matrix that contains normalized ranks of 6039 ORF’s in 34 samples.

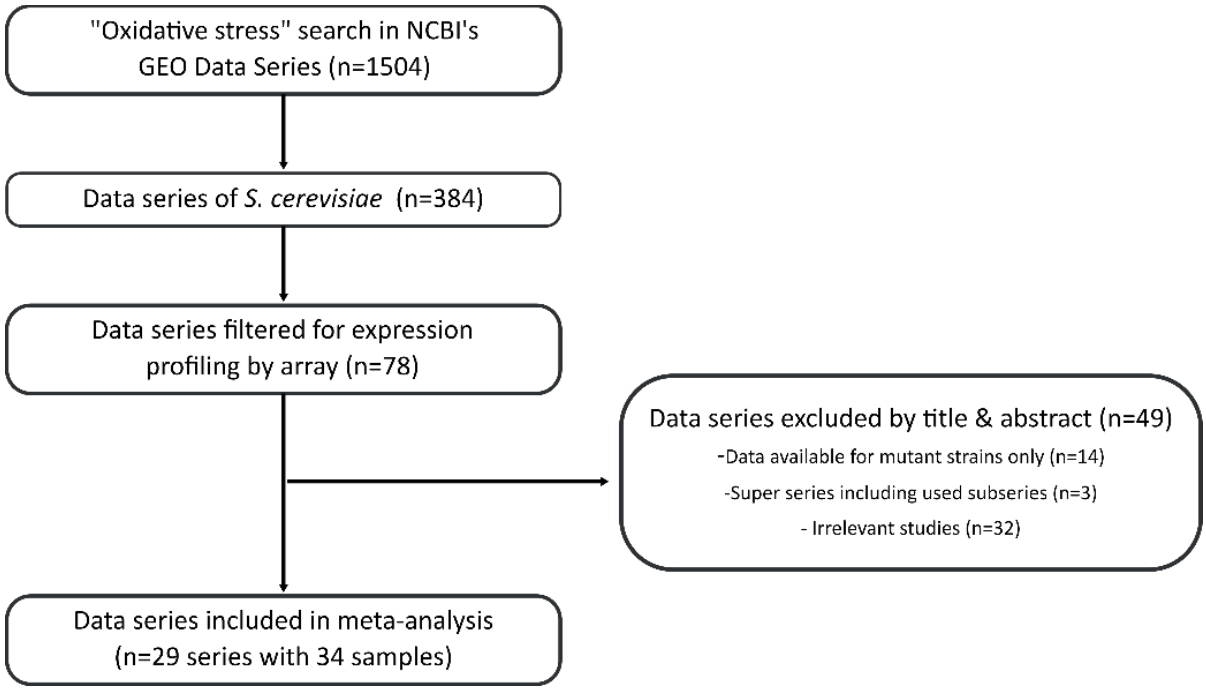

Figure 2. Transcriptome data acquisition workflow to compile the data used in this study.

Aggregation of transcriptome data

The aggregation of transcriptome data is performed using Robust Rank Aggregation algorithm and the R package RobustRankAggreg (Kolde et al., 2012). In case the transcript level for a specific probe, the corresponding entry to the rank matrix was replaced with maximum normalized rank value, namely 1. The rank matrix was aggregated using aggregateRanks function, which calculates ρ-scores for each transcript and sorts them in increasing order.

Functional analysis of differentially expressed genes in aggregated list

GO and KEGG Enrichment

For the ORF's that are overrepresented in all datasets are determined based on their ρ-scores, and a cutoff-score of 0.1. For overrepresented genes ($\rho < 0.1$), gene set enrichment analysis based on Gene Onthology (GO) and KEGG pathways were performed using R packages clusterProfiler and org.Sc.sgd.db Bioconductor genome wide annotation for yeast, and DAVID Gene Functional Annotation and Classification Tools (https://david.ncifcrf.gov/home.jsp) and AmiGO database (Carbon et al., 2009). For GO annotation for all GO ontologies, molecular function, bioprocess and cellular component, were performed using enrichGO function using Ensembl ID's as key types, false discovery rate as p value adjustment method and p-value cutoff was set to 0.1 (same in KEGG enrichment). The KEGG annotation was performed similarly using NCBI Gene ID's as keys using enrichKEGG function.

Results and Discussion

This study focuses on finding common and significant gene sets, differentially expressed upon oxidative stress, using meta-analysis of transcriptome data, collected from public databases. The robust rank aggregation method used here allows to overrepresented genes. It should be noted that the final set of genes may or may not be the most differentially expressed all together in a single dataset, rather, the final set of genes are differentially expressed in most (evaluated with statistically significance) of the datasets considered. The overall workflow for selection of microarray transcriptome datasets is given in Figure 2. The selected final datasets with 34 samples include direct or indirect induction of oxidative stress response on wild-type strains, while studies including mutant strains, investigating unconventional conditions such as radioactive materials or freezing were not considered. This way, more focused experimental context was obtained.

DEG analysis was *de novo* performed for series containing replicates or series where raw data is available in .CEL, .txt or .gpr formats. The ranking of transcripts was performed based on absolute logFC, so up- or downregulation was not separately investigated in aggregated list. Normalization of the ranks considering the number of the differentially expressed genes allows lowering the effect of unreliable DEG lists. In case, the raw data or replicates are not available in a specific dataset, statistical significance of the differential expression cannot be known, yet, published information on logFC is used to obtain statistically significant aggregated ranks.

Upon DEG analysis for each sample, the normalized rank lists from each sample were merged to obtain 10715x34 rank matrix (10715 ORF's and 34 samples) containing normalized rank list, one for each sample. Following the work of Kolde 2012, p-values for beta distribution were calculated (and corrected for possible false positives due to multiple hypothesis testing) for each transcript. Finally, 83 ORF's have ρ-score less than 1 and 42 of these were considered to be overrepresented in all data set ($\rho < 0.1$, Table 2).

Oxidative stress might be induced directly by perturbing the culture with e.g. a hyperoxide or indirectly using excess aeration. To assess whether the stress induction method delivers different set of genes or not, a subset of 10 DEG lists that contains only direct induction methods by H_2O_2, LoaOOH and CHP, were aggregated. Comparison of the results were shown in Figure 3. In this case, 23 of the 10715 ORF's had ρ-score less than 0.1. Interestingly, the 23 ORFs were not a subset of the initial list 43 ORF's obtained from the dataset with 34 samples, though several genes are shared between these two lists (Figure 3).

Table 2. ρ-scores of significantly differentially expressed genes in analyses containing all samples and only samples with direct induction of oxidative stress.

ORF	Description	*ρ Score* (All Samples)	*ρ Score* (Direct induction)
YPL171C	NADPH dehydrogenase	2.80E-09	2.26E-06
YKL071W	NADH-dependent aldehyde reductase	1.14E-08	0.001887
YKL086W	sulfiredoxin	6.72E-08	6.57E-06
YDR453C	thioredoxin peroxidase TSA2	2.07E-06	9.28E-05
YOL052C-A	Ddr2p, Multi-stress response protein	1.54E-05	0.017453
YBR072W	chaperone protein HSP26	3.25E-05	0.084638
YCR021C	Hsp30p	4.89E-05	0.043349
YMR090W	Putative protein of unknown function	6.28E-05	-
YLL060C	glutathione transferase GTT2	2.54E-04	0.006030
YOR382W	Fit2p	2.83E-04	0.003935
YHR048W	Yhk8p	3.05E-04	1.12E-04
YER103W	Hsp70 family chaperone SSA4	4.07E-04	-
YHR087W	Rtc3p	0.000776	0.044299
YCL026C-A	type II nitroreductase	0.001375	0.003311
YLR327C	Tma10p	0.001497	-
YGR248W	6-phosphogluconolactonase SOL4	0.002262	0.071480
YML131W	Protein of unknown function	0.002967	-
YKR076W	S-glutathionyl-(chloro)hydroquinone reductase	0.003412	0.035562
YDL243C	putative aryl-alcohol dehydrogenase	0.005133	-
YGR088W	catalase T	0.005402	
YFL053W	dihydroxyacetone kinase	0.006326	0.024013
YBR244W	glutathione peroxidase GPX2	0.007829	0.015116
YGR224W	azole transporter	0.007841	-
YER067W	Rgi1p	0.008506	-
YFL014W	lipid-binding protein HSP12	0.012983	-
YMR173W	DNA damage-responsive protein 48	0.019120	0.069446
YFL056C	Putative aryl-alcohol dehydrogenase	0.023097	-
YDR171W	heat shock protein HSP42	0.023735	-
YNL194C	Integral membrane protein	0.026158	-
YDR256C	catalase A	0.028125	0.093993
YLR205C	Hmx1p	0.031368	0.011914
YDL204W	Rtn2p	0.035241	-
YGR008C	ATPase-stabilizing factor family protein	0.036289	-

Table 2. *(continued)*

YML128C	Msc1p	0.038967	-
YLR346C	Cis1p	0.045650	-
YHR139C	Sps100p	0.046043	0.054276
YDR533C	glutathione-independent methylglyoxalase	0.050285	-
YOL151W	methylglyoxal reductase (NADPH-dependent) GRE2	0.056597	0.040635
YGR035C	Putative protein of unknown function,	0.065623	-
YDL246C	L-iditol 2-dehydrogenase SOR2	0.071729	-
YKL070W	uncharacterized protein	0.095622	-
YLR136C	Tis11p	-	0.037149
YFR053C	hexokinase 1	-	0.050526
YBR047W	Fmp23p	-	0.054616
YMR011W	hexose transporter HXT2	-	0.072490
YJR005C-A	Lso1p	-	0.097781
YPR030W	Csr2p	-	0.098063

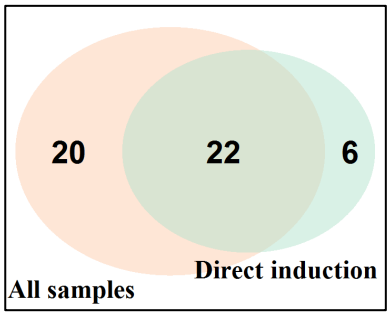

Figure 3. Venn diagram of the number of ORF's that are differentially expressed in aggregated list of all samples and the samples that were obtained from direct induction experiments with replicates.

To gain further insights on the functions of the aggregated list of genes, gene set enrichment analysis based on GO-terms was performed. Enriched GO-terms in the resulting gene set in terms of biological process and molecular function are presented in Figure 4a and 4b. Most overrepresented GO-terms are oxidative stress (30% of the genes), cellular response to chemical stimulus (36% of the genes) and oxidation-reduction process (40% of the genes) for both "all samples" and "direct oxidative stress method samples" lists.

Interestingly, 2 genes in both lists are annotated to be involved in glutathione metabolic process. Differences also exists in enriched GO-terms, in both lists. In direct induction list, 5 (out of 28) genes are annotated in chemical homeostasis and 2 genes are annotated in fructose/glucose transmembrane transport. In molecular function, similar GO-term landscape is observed. In "all samples list", 14 genes are annotated in oxidoreductase activity, 2 genes in glutathione reductase activity. Lastly for cellular component, 60% of the ORFs were located in cytoplasm, 15% in cell periphery and 18% in plasma membrane. Further manual annotation using Yeast Genome (https://www.yeastgenome.org) website resulted in 16 manually curated GO-cellular component annotation, 8 in cytoplasm and 5 in nucleus.

To get more insight into gene sets, we further focused on the set of genes in two GO terms, (i) glutathione metabolic process (GO:0006749) and response to oxidative stress (GO:0006979), containing 19 ORF's and 125 ORF's respectively according to AmiGO. For those ORFs, the ρ-scores were plotted, showing the count of the samples that the specific ORF was differentially expressed (Figure 5). For glutathione metabolic process, 18 of the genes were found to be differentially expressed in more than two samples, but only two of them YKR076W (*ECM4*, differentially expressed in 18 samples) and YLL060C (*GTT2*, differentially expressed in 20 samples) were found to be significantly different in all samples. For response to oxidative stress, twelve of 125 genes were found to be significantly differentially expressed in all samples (average number of samples that these genes were found to be differentially expressed 16).

Upon aggregation, 28 genes were found to be significantly overrepresented in the lists of differential expressed genes from individual experiments. These genes are categorized as responsible for antioxidant capacity, oxidoreductase activity (acting on peroxide as acceptor, acting on a sulfur group of donors, and acting on NAD(P)H), peroxidase mechanisms, glutathione transferase activity, peroxiredoxin mechanisms, heme binding and tetrapyrrole binding activities. The majority of the above stated functions are related to the electron transfers or mechanisms for reducing the oxidative stress.

These genes are further inspected in KEGG database to further probe their biological context. Significant hits are observed at longevity regulating pathway, carbon metabolism, tryptophan metabolism, fructose and mannose metabolism, protein processing in endoplasmic reticulum, glyoxylate and dicarboxylate metabolism, MAPK signaling pathway – yeast,

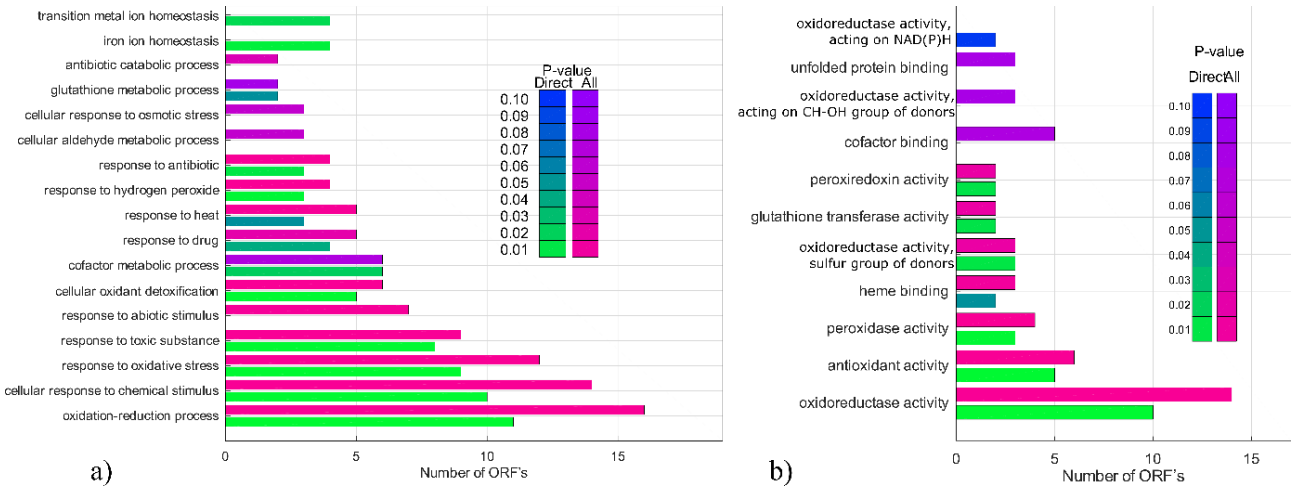

Figure 4. GO enrichment results for all list of samples which direct induction applied **(a)** GO-Bioprocess **(b)** GO-Molecular function.

peroxisome, pyruvate metabolism, pentose and glucuronate interconversions and propanoate metabolism. Interestingly, when the focused dataset (direct oxidative stress), carbon metabolism and fructose and mannose metabolism are the main hits metabolic pathways, especially with hydrogen peroxide-induced oxidative stress, aiming at increasing NADPH, an important cofactor in the oxidation reduction mechanisms (glutaredoxins, thioredoxins). Also, trehalose synthesis and synthesis of three enzymes (glucose-6-phosphate dehydrogenase, transketolases and transaldolase) of pentose phosphate pathways induced (Godon et al., 1998) overall increasing NADPH levels.

Next to cofactor balances, oxidative stress also induces antioxidant defense mechanisms, a protein degradation pathway. Glutathione plays a role against oxidative stress in yeast both as a metabolite in non-enzymatic mechanisms and as a cofactor in enzymatic mechanisms (glutathione peroxidase, glutathione

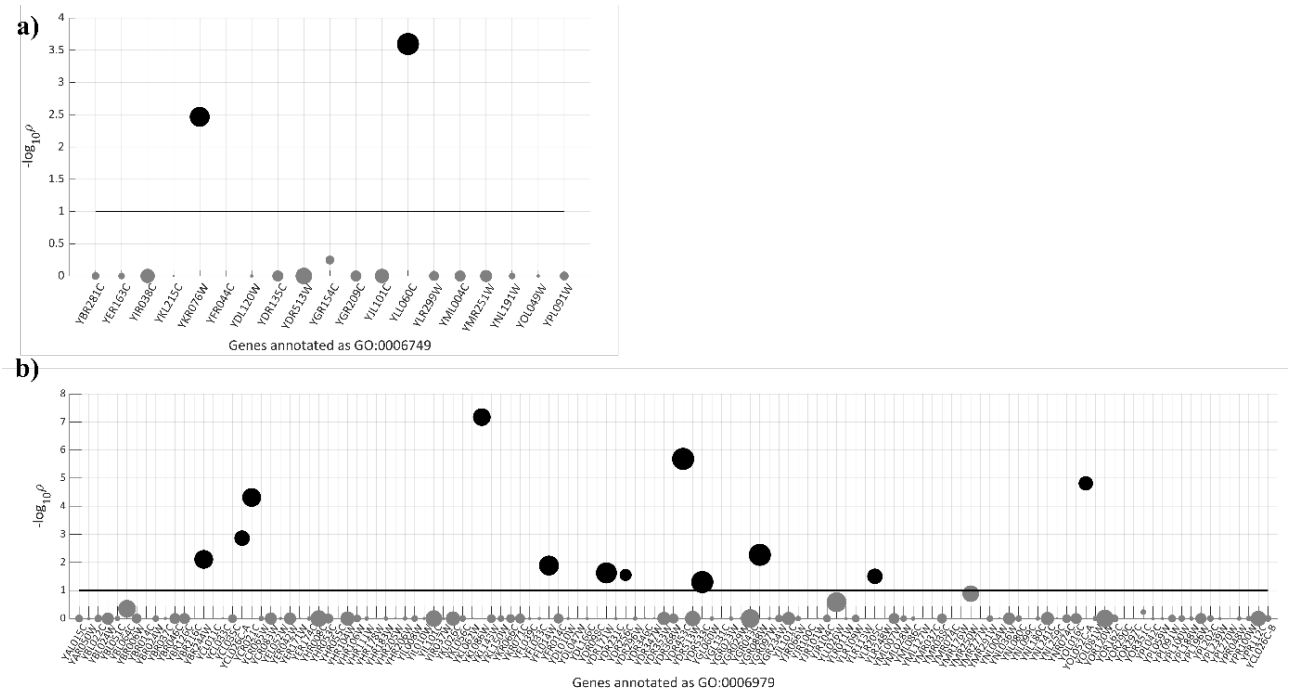

Figure 1. **(a)** Rank scores of ORF's involved in glutathione metabolic process. ORF's that differentially expressed were shown with black dots, line represents significance level (rho=0.1) and marker sizes refer to number of samples that these ORF's were found to be differentially expressed. **(b)** Rank scores of ORF's involved in response to oxidative stress. ORF's that differentially expressed were shown with black dots, line represents significance level (ρ=0.1) and marker sizes refer to number of samples that these ORF's were found to be differentially expressed.

reductase (Douglas, 1987, Meister, 1988). The protein degradation via H_2O_2–induced oxidative stress causing oxidation of major proteins (pyruvate decarboxylase, fatty acid synthase, and glyceraldehyde-3-phosphate dehydrogenase (Tdh) are also separately reported in literature (Cabiscol et al., 2000).

This work combines several datasets related to oxidative stress in yeast. It should be noted that a typical extension is a detailed analysis that would require careful grouping taking into account each stressor as well as corresponding (known) defense or response mechanisms and intracellular indicators (protein carbonylation, Thiobarbituric Acid Reactive Substances (TBARS) etc.). In selecting the datasets (Figure 2), we adopt a top-down approach and care is taken not to "over-slice" the data by too-detailed-subgrouping, allowing not to lose general features.

In performing meta-analysis studies, one expected pitfall is on dosage experiments, where the level of stressor triggers different or additional response/defense mechanisms. In that case, the ranking of a selected transcript will like change. However, considering nearly 6000 all genes in yeast, it would be highly unlikely to find such a finely tuned transcript that would be common key to all experiments due to robustness of metabolism. One specific transcript will be of key importance of that specific, isolated experiment, and is expected to be diluted over other experiments. In contrast, the transcripts in first couple of rows in Table 2 are found nearly always among top 20 differentially expressed genes.

Conclusion

Identification of key processes as a response to environmental stress is one of the key problems in biology, not only for our fundamental understanding but also to rational design of improved industrial strains and processes. Focusing on oxidative stress response, robust rank aggregation was performed to extract useful information from various experiments as individual experiments uses different techniques, have different agenda and are performed by different labs. This work strived for finding metabolic engineering targets for improved yields and productivities of industrially important microorganisms. Analysis underlined that key processes center around carbon, redox and glutathione metabolism and pointed interplay among those. In conclusion, this study illustrates bioinformatics analysis on capturing common features in a large, aggregated datasets and points to key common features and further metabolic engineering targets. Further investigation on distribution and possible redirection of metabolic fluxes allow further pinpointing key reactions.

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BIOTECH STUDIES

REVIEW

Antiviral mechanisms related to lactic acid bacteria and fermented food products

Zümrüt Begüm ÖGEL[1,*], Hale İnci ÖZTÜRK[1]

[1]Department of Food Engineering, Konya Food and Agriculture University, 42080 Meram, Konya, Turkey.

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***Corresponding Author**
Tel.: +90 532 627 9162
E-mail:
zumrut.ogel@gidatarim.edu.tr

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Abstract
The Covid-19 pandemics laid stress on the significance of having a strong immune system in coping with viral infections. Nutrition is important in the modulation of our immune systems. Recent studies have shown that probiotics, most of which are lactic acid bacteria (LAB) naturally present in fermented food products, can boost the immune system of their host. Although responses are generally strain and dose dependent, in one way or another, most LAB are capable of enhancing both the innate and the adaptive immune responses in animal model systems. In addition to their ability of boosting the immune system, LAB directly or indirectly by means of the fermentation process, can generate bioactive metabolites having antiviral properties, such as peptides. LAB are shown to have antiviral mechanisms that affect both upper respiratory tract and gastrointestinal viral infections. Not only live cells but also heat-killed cells of probiotics (paraprobiotics) are shown to be effective. These wide range of antiviral mechanisms suggest that the diversity of LAB in the food product is likely to enhance the variety and strength of health benefits obtained from fermented foods. Traditional fermented foods have significantly higher microbiodiversity with respect to the LAB species, as compared to those produced by commercial cultures. This is particularly valid for the Lactobacilli, where several species and strains have proven to be antiviral probiotics and are natural inhabitants of fermented foods at the same time. While drawing attention to the antiviral properties of both live and dead cells of LAB, this review aims to underline the significance of supporting our health with the wealth of foods that are rich in terms of their microbial diversity. Further scientific research must focus on the several technical, biological, and clinical aspects of traditional fermentations.

Introduction

Probiotics are defined as 'Live microorganisms which when administered in adequate amounts confer a health benefit on the host' (FAO/WHO, 2006). The early *in vitro* tests for the study of probiotic strains included resistance to gastric acidity, bile acid resistance, adherence to mucus and/or human epithelial cells and cell lines, antimicrobial activity against potentially pathogenic bacteria, ability to reduce pathogen adhesion to surfaces, bile salt hydrolase activity and resistance to spermicides (applicable to probiotics for vaginal use) (FAO/WHO, 2006). These criteria were based on the belief that probiotic strains have to survive the harsh conditions of the stomach and pass to the intestines at sufficient quantities, in order to be effective. Yet, advances in research on probiotics have shown that a probiotic organism may function at locations other than the intestines, such as the pharynx (Santagati et al., 2015) and by a wide range of mechanisms which may be by direct contact with the pathogen, or indirectly, for instance, by boosting the host's immune system. Accordingly, it was agreed that probiotic organisms (single or in combination) should provide convincing evidence regarding a certain health-benefit by well-conducted studies in humans, including: positive meta-analyses on specific strain(s) or strain combinations on a diversity of clinical endpoints such as infectious diarrhea, antibiotic-associated diarrhea, irritable bowel syndrome and ulcerative colitis (AlFaleh et al., 2011; Allen et al., 2010; Aponte et al., 2013; Goldenberg et al., 2017; Ritchie & Romanuk, 2012), by scientifically accepted assessment methods, well-conducted RCTs (Randomized Controlled Trials) or strong evidence from large observational studies (Hill et al., 2014).

In general, probiotic organisms are mostly lactic acid bacteria that occur in fermented food products. Health effects of probiotics are generally at the strain level and in a dose-dependent manner. However, in some countries, such as Canada certain species of *Bifidobacterium* and *Lactobacillus*, when delivered in food at a level of 1 × 109 colony forming units (CFU) per serving, have been accepted as being probiotic, resulting in a strain-independent assignment of some bacteria as probiotics (Hill et al., 2014).

Despite of all the health benefits of probiotics, it is now clear that the use of probiotics is not safe for all the individuals, in particular those who are immunodeficient (Kothari et al., 2019; Wagner & Balish, 1998). The possibility that some probiotics might become opportunistic pathogens is surely a disadvantage for the consumption of live probiotics as supplements. Hence, it is now suggested that the consumption of capsules with live probiotics should be regarded as drug consumption and should, therefore be much more strictly regulated (Kothari et al., 2019). While the safety of probiotics is being questioned, recent studies have shown that even the dead cells of probiotics might have health benefits, especially through the modulation of the immune system (Taverniti & Guglielmetti, 2011). Since the definition of probiotics requires that cells are viable, a new term for the non-viable forms of probiotics has been proposed as the 'paraprobiotics' (Taverniti & Guglielmetti, 2011).

The concept of paraprobiotics is important, not only because it provides a safer alternative to the use of viable microorganisms, but also because it raises the question as to how appropriate it is to define probiotics as 'live microorganisms'. It further raises the question as to the best form of probiotic consumption in terms of safety and health benefits. Namely, is it better to use high numbers of a few different viable microorganisms as in the case of capsules, or is it better to have a greater variety of different species at lower cell counts, as in the case of fermented foods, which are also likely to be rich in terms of paraprobiotics? Is it better to use commercially cultured fermented food products or traditional fermented foods that generally contain a broader range of microbial cultures, etc. (Rezac et al., 2018)? These questions need to be answered by further research.

Antiviral Mechanisms

The importance of probiotics, most being lactic acid bacteria (LAB), in both preventing and/or alleviating the symptoms or the duration of viral diseases is of growing importance (Al Kassaa, 2017). The reader is referred to excellent recent reviews on the topic (Al Kassaa et al., 2014; Kanauchi et al., 2018; Lehtoranta et al., 2014; Sousa et al., 2020; Tapiovaara et al., 2016).

Viral infections are either upper respiratory tract infections or viral gastroenteritis. Interestingly, probiotics have mechanisms that can act on both groups of viral infections. The antiviral mechanisms of probiotic LAB are summarized in Figure 1.

These mechanisms can be classified into two major categories: 1) Mechanisms related directly to the

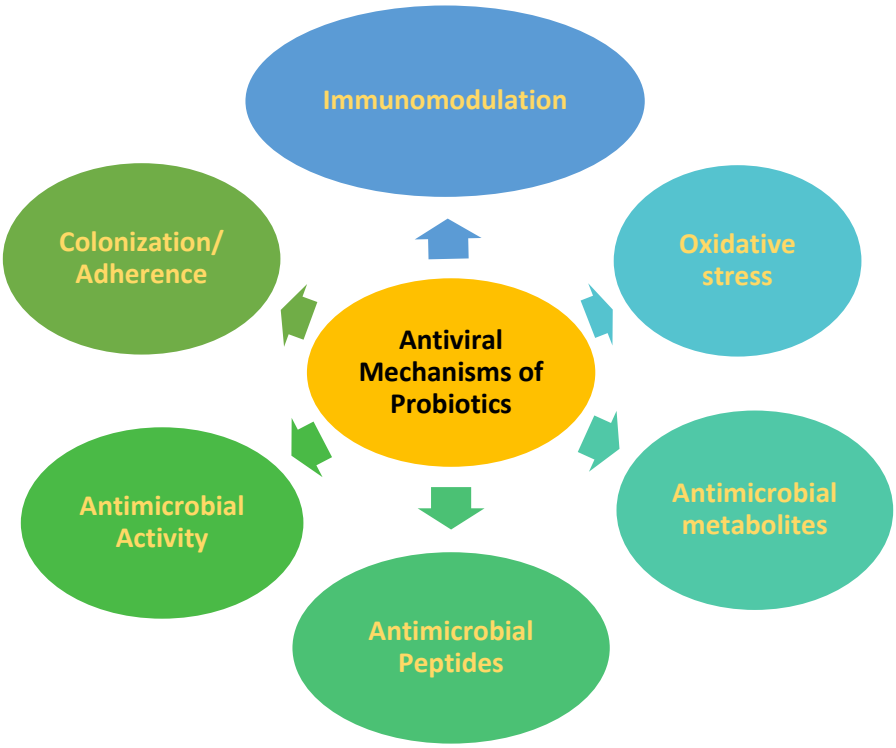

Figure 1. Summary of the various antiviral mechanisms of probiotics.

organism itself and its cross-talk with the host and/or contact with the virus. Such probiotic LAB may originate from the food, or they may be supplemented in the form of capsules. 2) Mechanisms related to the antiviral metabolites generated in food products fermented by LAB. In such cases, although the organism might be the same, metabolites might differ due to differences in the fermented food material, such as milk, cereals, legumes, or vegetables. Based on this classification, only the first group of antiviral mechanisms will function when probiotics are consumed in the form of capsules, whereas in the case of fermented foods, both mechanisms will act together, including those related to the existence of paraprobiotics (Kanauchi et al., 2018).

Regarding the site of antiviral action of probiotic LAB, the intestines are often mentioned. However, research indicates that intestines are not the only site of antiviral action. The gastrointestinal (GI) tract starts at the mouth and ends at the anus. The mouth/nose is the first part of the GI that comes in contact with the environment. When probiotics are administered in the form of tablets, they pass directly to the stomach. However, when probiotic food products are consumed, there is contact with the mouth. Based on research this fact appears to be significant due to two reasons; probiotics can colonize the tonsils and the pharynx for several hours to several days, thereby exerting their actions directly at the first contact site of the GI. For example, in a study conducted by Santagati et al. (2015), nasal sprays of *Streptococcus* (S.) *salivarius* 24SMBc were shown to have the capability of colonizing the rhinopharynx tissues in 95% of subjects, aged 30-54 years, and persisted in 55% of the individuals even after 6 days. In another study, where *Lactobacillus (Lb.) plantarum* DSM 9843 was administered to healthy volunteers who either gargled or drank oatmeal gruel with or without fruit juice, *Lb. plantarum* DSM 9843 was found in tonsillar scrapings 4–8 h after intake, suggesting an ability of these bacteria to adhere to tonsillar cells (Stjernquist-Desatnik, 2000).

Accordingly, studies indicate that the antiviral effect of probiotic LAB can be initiated in the mouth, tonsils, and the pharynx. These observations are important because they may suggest a greater advantage of probiotic food consumption in the prevention of URT viral infections as compared to probiotics intake in the form of capsules, especially regarding mechanisms that involve direct contact of the probiotic organisms with the infectious viruses before they can reach the lungs.

Among the various antiviral mechanisms, perhaps the immune boosting effect of probiotic LAB is the most important one. As opposed to bacterial infections, viruses cannot be eliminated by the use of antibiotics. Although medications target viral proteins/enzymes for inactivation, most of these medications only have limited effect in alleviating symptoms and some have side-effects. At the end of the day, the most effective antiviral mechanisms are those related to the modulation of the host's immune system. As we have all seen in the case of Covid-19, those individuals with a strong immune system could cope with the virus with almost no symptoms at all, while those, especially the elderly people, with weak immune systems ended up having very severe symptoms.

The immunomodulatory effect of probiotics, and perhaps of most lactic acid bacteria present in fermented food products, involves the following: protection of epithelial cells by stimulating the production of mucin and the secretion of mucus; biofilm formation which probably saturates the receptors, including viral receptors; activation of dendritic cells to secrete proinflammatory cytokines, such as IL-6, IL-12 and interferon (IFN)γ; boosting the innate immune cells, such as macrophages and natural killer (NK) cells that kill viruses and virus-infected cells, produce interferon IFNα, an antiviral cytokine; stimulating the overexpression of innate immunity-related genes such as Toll-like receptors (TLR7) (Al Kassaa, 2017; Pimentel-Nunes et al., 2010). Some of these mechanisms occur in the lungs, upon infection by respiratory viruses, while the probiotics thrive in the gut (Al Kassaa, 2017). The LAB are also known to enhance the adaptive immune response, including lymphocyte proliferation and GI tract specific IgA production and others (Tsai et al., 2012).

Another antiviral mechanism of probiotics is related to the production bioactive peptides. These peptides can be generated either by the probiotic organism itself or generated by the enzymatic digestion of proteins that are part of the food source; the latter is relevant to all lactic acid bacteria that have the capability of digesting food proteins with proteases.

Bioactive peptides are functional peptides consisting of small peptide sequences, such as 2-20 amino acid residues, that have important roles in human health (Hartmann & Meisel, 2007). Although milk and dairy products are rich food sources in terms of bioactive peptides, these peptides are also found in most plants, various meat types, and eggs (Korhonen & Pihlanto, 2006; Pihlanto & Korhonen, 2015). These peptides originate from the hydrolysis of the native proteins of the food source, and have been revealed to have functional properties such as opioid, mineral-binding, immunomodulatory, antimicrobial, antioxidant, antithrombotic, hypocholesterolemic, anticarcinogenic, etc. (Korhonen & Pihlanto, 2006; McClements et al., 2009; Meisel, 2005). They are either formed by the activity of their native enzymes or by microbial proteolytic enzymes. Especially in fermented foods, proteolytic enzymes belonging to starter cultures or natural microbiota are responsible for the formation of bioactive peptides (Michaelidou, 2008; Szwajkowska et al., 2011). Bioactive peptides with different lengths of amino acids were obtained by the activity of bacterial peptidases and proteases (Ardö et al., 2017). Moreover, intracellular enzymes that are released as a result of autolysis of dead bacterial cells may also play a role in

the formation of bioactive peptides (Fan et al., 2019; Jensen et al., 2009; Solieri et al., 2018). The latter example suggests that the concept of paraprobiotics may be significant in fermented food products, particularly for those products undergoing storage in brine or those kept under long periods of storage for ripening, etc.

Although most studies on the functional properties of bioactive peptides in fermented products have focused on antibacterial peptides, there are some studies on the antiviral properties of both proteins in some foods and peptides formed by their enzymatic hydrolysis. Mandal et al. (2016) have informed that bioactive peptides generally bind to cell receptors of host, inhibiting the attachment of viruses. Bioactive peptides produced by the enzymatic activities of probiotics can also show the antiviral effect by directly binding to virus particles. Furthermore, bacteriocins, which are peptides synthesized in the ribosomes of bacteria, can have antiviral effects by aggregating of virus particles, blocking the receptor sites where viruses are attached in the host cell, or inhibiting key reactions in the replication cycle of viruses (Todorov et al., 2010; Wachsman et al., 2003).

Among the various metabolites of probiotics, hydrogen peroxide and lactic acid produced by *Lactobacillus* species were shown to play an important role in their antiviral effects. Conti et al. (2009) suggested that lactic acid and H_2O_2 could be responsible for reducing intracellular events of herpes simplex virus type 2 multiplication. Acidic pH ≤ 4.5, which occurs due to lactic acid production, disrupts herpes simplex virus type 2 and leads to a reduction in attachment and invasion of this virus (Tuyama et al., 2006). In addition, exopolysaccharides (EPS) produced by probiotic bacteria have been reported to have antiviral effects in several studies (Arena et al., 2006; Kanmani et al., 2018; Kim et al., 2018). The EPS are known to stimulate immunologic system, to prevent the absorption and penetration of viruses into host cell, and to inhibit various retroviral reverse transcriptase (Arena et al., 2006; Saadat et al., 2019). EPS produced by *Lb. delbrueckii* OLL1073R-1 stimulated type I interferon production (Kanmani et al., 2018). Also, EPS of *Lb. plantarum* LRCC310 were presented to have an inhibition effect against human rotavirus Wa strain *in vitro* and they also were found to decrease the rotavirus infection in mice models (Kim et al., 2018).

Antiviral Probiotic Lactic Acid Bacteria

Lactic acid bacteria (LAB) or probiotics can inhibit viruses in a variety of ways. These include direct physical interaction between viruses and bacteria, stimulation of the host's immune system, and the production of antiviral substances with or without protein structure (Al Kassaa et al., 2014). Direct interactions prevent adsorption of the virus into cells. In some experimental observations, it has been reported that various probiotic bacteria inhibit some viruses by direct binding (Al Kassaa et al., 2014; Botić et al., 2007; Z. Wang et al., 2013). Direct interactions between vesicular stomatitis virus and probiotic bacteria including *Bifidobacterium* (B.) *breve* DSM 20091, *B. longum* Q 46, *Lb. reuteri* DSM 12246, *Lb. plantarum* M1.1, *Lb. paracasei* F19, *Lb. paracasei* A14, and *Lb. paracasei/rhamnosus* Q 85 (Botić et al., 2007), between influenza viruses and *Enterococcus* (E.) *faecium* NCIMB 10415 (Z. Wang et al., 2013), and herpes simplex type 2 and *Lb. gasseri* CMUL57 (Al Kassaa et al., 2014) have been observed.

The substances such as lipoteichoic acid, peptidoglycan, nucleic acids, and muramyl dipeptide found in probiotics have a stimulating effect on the immune system (Pimentel-Nunes et al., 2010). Probiotic bacteria stimulate immune system by regulating T cells, lymphoid cells, and T helper 17 cells by the recognition of probiotics themselves or their metabolic products (Kanauchi et al., 2018). It has been suggested that the great majority of probiotic bacteria show inhibitory activity against most important respiratory viruses with their immunomodulatory systems (Lehtoranta et al., 2014). Since these viruses attach to mucosal cells in the respiratory tract, probiotics and their antimicrobial agents cannot physically interact with the virus (Al Kassaa, 2017). Therefore, probiotics eliminate these viruses by stimulating the host's immune system. Therefore, these microorganisms are also called immunobiotics (Kitazawa et al., 2013).

Probiotic LAB and upper respiratory tract viruses

Among the probiotics, the antiviral effect of Lactobacilli against respiratory viruses is most widely examined. Fermented milk product containing *Lb. casei* DN-114 001 strain has been reported to reduce respiratory infections by causing an increase in neutrophil, leukocyte, and natural killer cell counts in humans (Guillemard et al., 2010). Moreover, yogurts fermented with *Lb. delbrueckii* subsp. *bulgaricus* OLL1073R-1 improved natural killer cell activity in elderly individuals (Makino et al., 2010). In a study performed on mice, *Lb. plantarum* L-137 strain was observed to increase T-helper-1 (Th1) production against influenza virus (H1N1) (Maeda et al., 2009). It was also determined that *Lb. plantarum* YU strain suppressed the proliferation of the H1N1 virus in the lungs of the mice by increasing the Th1 immune response (Kawashima et al., 2011).

In general, it appears that IFN-α takes a crucial role in the antiviral immune response by triggering the cytotoxic activity of NK cells (Kanauchi et al., 2018). For example, *Lb. acidophilus* L-92 antiviral activity against influenza A/PR/8/34 (H1N1) virus occurred by increasing NK cell and the production of IFN-α in a mouse model (Goto et al., 2013). *Lb. rhamnosus* Lr05 and Lr06 strains provided a protective effect against human respiratory virus strain A2 by stimulating the production of interferon (IFN)-α, IFN-β, and interleukin (IL)-6 (Tomosada et al., 2013). In another study on mice

model, *Lb. brevis* KB-290 also presented inhibition activity towards influenza A/PR/8/34 virus by stimulating immunoglobulin A (IgA) and IFN-α (Waki et al., 2014). *Lactococcus lactis* JCM 5805 strain inactivated influenza A/PR/8/34 virus by generating high amounts of IFN-α in human (Sugimura et al., 2015). *Lb. gasseri* TMC0356 reduced influenza A/PR/8/34 symptoms in experimental mice by improving the secretion of IgA, IFN-γ, IL-12, and IL-6 (Kawase et al., 2010). *Lb. pentosus* S-PT84 strain inhibited influenza A/PR/8/34 virus by activating lung NK cells and modulating the Th1/Th2 balance (Izumo et al., 2010). On the other side, *Lb. fermentum* CJL-112 strain augmented IL-2 and IFN-γ cytokines against influenza A/NWS/33 (H1N1) virus (Yeo et al., 2014).

Studies on Bifidobacteria have shown immunomodulation similar to those of Lactobacilli. *B. longum* stimulated the production of interleukins (IL) and IFN cytokines against influenza A/PR/8/34 virus (Iwabuchi et al., 2011). *B. animalis* subsp. lactis Bb12 in breastfed infants was found to increase anti-poliovirus specific IgA, but not anti-rotavirus specific IgA (Holscher et al., 2012). Various probiotic strains can be used in combination to provide a more effective antiviral property.

Probiotic LAB and gastrointestinal viruses

It is known that probiotics decline the duration of diarrhea caused by rotavirus. Probiotic application has been revealed to be more effective at the beginning of the diarrheal stage (Rosenfeldt et al., 2002). Probiotic *B. longum* and *Lb. acidophilus* strains showed antiviral activity against rotavirus *in vitro* and they reduced the duration of diarrhea in pediatric patients (Lee et al., 2015). In addition to this, it was determined that the duration of rotavirus diarrhea decreased as a result of giving the solution containing probiotic *Lb. acidophilus, Lb. rhamnosus, B. longum* and *Saccharomyces boulardii* strains orally to children under 2 years of age (Grandy et al., 2010). Similarly, *Lb. rhamnosus* 19070-2 and *Lb. reuteri* DSM 12246 reduced length of hospital stay based on diarrhea (Rosenfeldt et al., 2002).

Cell-free supernatants of yogurt containing *Lb. acidophilus*, *Lb. rhamnosus*, *Lb. plantarum*, *S. thermophilus* and *B. bifidum* was found to be more effective than that of MRS broth containing these strains against enteroviruses (Choi et al., 2009). This inhibitory effect probably occurred due to antiviral substances produced by bacterial cells. Considering the result of this study, the intake of probiotics with foods may be more advantageous than taking them in capsules or tablets.

Antiviral proteinaceous compounds produced by probiotic LAB

Bacteriocins are antibacterial peptides synthesized by bacteria in their ribosome (Todorov et al., 2010). Al Kassaa et al. (2014) have been asserted that some bacteriocins have both antibacterial and antiviral properties. Various bacteria including probiotics can produce such antiviral peptides. In published studies, subtilosin bacteriocin of *Bacillus amyloliquefaciens*, bacteriocin of *Lb. delbrueckii*, TVAAPSVFIFPPSDEQLK and EAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSK bacteriocins of *Bacillus subtilis*, bacteriocin of *Lb. bulgaricus* 761 N, bacteriocin of *E. mundtii* ST4V, pediocin-like bacteriocin of *E. faecium* and enterocin AAR-71 of *E. faecalis* AAR-71 showed both antibacterial and antiviral activities (Al Kassaa et al., 2014; Todorov et al., 2005, 2010; Torres et al., 2013). Yang et al. (2017) have also reported that the metabolic products containing peptides of probiotic *Lb. plantarum* N4 strain have antiviral effects. The metabolic products, including acids, alcohols, peptides, esters, ketones, phenols, of probiotic *Lb. plantarum* N4 strain, has been found to be effective against gastroenteritis coronavirus (Yang et al., 2017). A pediocin-like bacteriocin produced by *E. faecium* ST5Ha strain has been reported to possess antiviral effect against HSV-1 (Todorov et al., 2010). The EC_{50} value of this bacteriocin against HSV-1 was found to be 50 μg/mL. It was determined that extracellular extracts, possibly including bacteriocins, of *Lb. bulgaricus* 761N strain showed antiviral activity against hepatitis C virus (HCV) (El-Adawi et al., 2015). *Lb. delbrueckii* subsp. *bulgaricus* 1043 was shown to be effective on influenza virus (Serkedjieva et al., 2000). As for bacteriocin of *E. mundtii,* it has inactivated herpes simplex viruses (1 and 2), a polio virus, and a measles virus depending on its dose (Todorov et al., 2005). The highest inhibition of this bacteriocin was found to show at a concentration of 400 μg/mL.

The antiviral actions of the various peptides derived by fermentations have been analysed. In a study on casein-derived peptides, although positively or negatively charged peptides from α_{s2}-, β- and κ-caseins had no antiviral effects (Floris et al., 2003), lactoferrin, a protein naturally found in milk, has been reported to have both antibacterial and antiviral properties (Farnaud & Evans, 2003; Pan et al., 2007). Additionally, ADRDQYELL and EDLIWK peptides produced by the enzymatic hydrolysis of lactoferrin showed antiviral properties (Dziuba et al., 2009).

Lactoferrin and lactoferrin derived peptides were also shown to present antiviral activity against HIV-1, poliovirus, rotavirus, herpes simplex virus, hepatitis C, G, and B viruses, and cytomegalovirus by increasing the immunological reactions in the host (Farnaud & Evans, 2003; Pan et al., 2007; Szwajkowska et al., 2011). Lactoferrin-derived ADRDQYELL and EDLIWK peptides have been revealed to show antiviral activity against herpes simplex virus type 1 (HSV1) (Siciliano et al., 1999). Additionally, κ-casein-derived glycomacropeptide and lactoferrin, which are originated from milk, have been reported to show activity against influenza virus (Kawasaki et al., 1993). These researchers attributed the inhibitory activity to sialic acids in glycomacropeptide and lactoferrin.

Immunomodulatory peptides can also act as antiviral agents because they regulate or improve immune responses. These biological active peptides have been identified in various fermented foods such as yogurt, cheese, fermented milk products (Fan et al., 2019; Jin et al., 2016; Pisanu et al., 2015; Tonolo et al., 2020). Pisanu et al. (2015) reported the presence of immunomodulator peptides in sheep cheese including YQEPVLGPVRGPFPI, YQEPVLGPVRGPFP, QEPVLGPVRGPFPIL, QEPVLGPVRGPFPI, QEPVLGPVRGPFP, QEPVLGPVRGPF, PVLGPVRGPFPIL, PVLGPVRGPFP, EPVLGPVRGPFPI, EPVLGPVRGPFP, EPVLGPVR, LGPVRGPFPI, GPVRGPFP, GPVRGPF, DMPIQAFLLYQEPVLGPVR peptides from the C terminal region of the β-casein and immunomodulator LNENLLR, VLNENLLR, LNENLLRF peptides from the N terminal region of the α_{s1}-casein. Besides, immunomodulator α_{s1}-casein derived peptide LNENLLRF and β-casein derived peptides GPVRGPFPI, EPVLGPVRGPFP, QEPVLGPVRGPFP, YQEPVLGPVRGPFP were determined in milk casein fermented by *Lb. helveticus* CICC6024. The α_{s1}-casein derived isracidin peptide (RPKHPIKHQGLPQEVLNENLLRF) is known to be an immunomodulating peptide. This peptide has been found in fermented milk products such as yogurt and cheese (Fontenele et al., 2017; Gagnaire et al., 2011; Jin et al., 2016). Therefore, fermented milk products appear to be a good source to benefit from these peptides.

Paraprobiotics

Observations on the health benefits of non-viable cells of probiotics has raised attention to a new concept of utilizing dead cells (paraprobiotics), instead of viable cells, as mentioned above. Results of studies comparing the efficacy of paraprobiotics and probiotics on the immune system are contradictory. Some studies have reported that living cells provide a stronger immune response as compared to dead cells (Cross et al., 2004; Haller et al., 2000). In contrast, some authors have asserted that the immuno-stimulatory effects of living and dead cells were similar (Lopez et al., 2008; Ryan et al., 2008). These differences may have occurred depending on the strain type and inactivation method used (Taverniti & Guglielmetti, 2011). In this context, more detailed studies comparing different strains and different inactivation methods are needed. Besides, the interaction of different strains and inactivation methods can be examined.

Fermented Food Products and The Rising Awareness on The Importance of Food Microbiodiversity

In October 2013, an expert panel was convened by the International Scientific Association for Probiotics and Prebiotics (ISAPP) to discuss the field of probiotics. Based on their proposal, there is evidence that supports the beneficial relationship between some foods containing live microbes, especially fermented dairy products, and reduced risk of certain diseases, such as type 2 diabetes (Hill et al., 2014). For example, one study conducted on >6,500 individuals found (Soedamah-Muthu et al., 2013) that yogurt consumers had reduced levels of circulating triglycerides and glucose, as well as reduced systolic blood pressure and insulin resistance, compared with non-consumers (H. Wang et al., 2013). However, the panel's judgement was towards excluding such food products from the list of probiotics and rather using the term 'live and active cultures' instead of 'probiotics'. This judgement was based on the fact that although such fermented food products might potentially have beneficial microbes, these cultures often represent a diverse community that is not well-defined in terms of strain composition and stability (Hill et al., 2014).

Traditional fermented foods are rich in terms of 'live and active cultures'. In other words, there are several different microbial species surviving together in the same environment (Dimidi et al., 2019). As compared to fermented foods produced from commercial probiotic and starter cultures, traditional fermented foods offer an extremely high microbial diversity. This microbiodiversity of natural fermented foods is valuable, considering that most probiotics are Lactobacilli that originate from these natural food environments. Thus, having a higher number of microbial species increases the likelihood of having microorganisms and biochemical reactions with health benefits. As the diversity of lactic acid bacteria increases, it can be suggested that the variety of antiviral as well as antibacterial and health promoting effects are likely to increase, although this has yet to be experimentally proven.

Fermented foods have been part of the Turkish Tradition for thousands of years. The Word 'Yogurt' is Turkish and is first mentioned in Divanü Lügati't-Türk written by Kaşgarlı Mahmut in the 10th century and by Balasagunlu Yusuf Hac Hacip in Kutadgu Bilig. Traditionally yogurt is used in healing food poisoning, diarrhea, and as an anti-toxin, in general. In Turkey, yogurt is industrially produced by *S. thermophilus* and *Lactobacillus delbrueckii* spp. *bulgaricus* cultures. However, still many people produce yogurt at home by traditional methods.

Kefir is another fermented dairy product frequently produced at home. Not only fermented milk products but also fermented vegetables are an important part of the Turkish traditional kitchen. For example, pickles from vegetables are routinely made at homes, allowing food preservation. Olives are also part of the widespread fermentation tradition.

The Turkish consumer believes that home made fermented foods are much healthier than the industrially produced ones. Although this is not strictly correct in terms of food hygiene, it is probably correct from a probiotic point-of-view. Although traditional fermented foods do not have a well-defined and standard microbial population, in general, all are rich in terms of their microbiodiversity. For example, for the

production of yogurt, milk is fermented with chickpea or by using herbs such as *Galium aparine* (Yogurt otu) etc. When yogurt is made in this traditional manner, namely by starting from natural resources of bacteria, the resulting yogurt consists of different bacterial species and is not limited by the two cultures, *S. thermophilus* and *Lb. bulgaricus*, described by the Turkish Food Codex (2009). In one study, Çebi and Aydın (2019) analysed lactic acid bacteria from fermented chickpea. Accordingly, *Lactoccoccus lactis* subsp*. lactis* was found as the dominant species (90%), followed by *Lb. plantarum* (6.7%) and *Lb. brevis* (3.3%). It was concluded that the natural microflora of chickpea was suitable for use in the preparation of sourdough. In another study, Hancıoğlu-Sıkılı (2003) isolated *Lb. plantarum, Lb. pentosus, Lb. sanfrancisco, E. mundtii, E. gallinarum, Pediococcus* (*P.*) *urinae-equi, Lb. viridencens,* and *S. thermophilus species* and *Saccharomyces cerevisiae* from chickpea. Studies on another popular Turkish fermented food product, namely Tarhana, has shown that the bacterial population consisted of *P. acidilactici* (27%), *S. thermophilus* (19%), *Lb. fermentum* (19%), *E. faecium* (12%), *P. pentosaceus* (7%), *Leuconostoc pseudomesenteroides* (5%), *Weissella cibaria* (4%), 2% each of *Lb. plantarum, Lb. delbrueckii spp. bulgaricus, Leuconostoc citreum*, 1% as *Lb. paraplantarum* and 0.5% *Lb. casei* (Sengun et al*.,* 2009). Kefir is yet another highly popular fermented dairy product in Turkey. Various studies have been conducted on the identification of microorganisms from kefir grains by culture-dependent and culture-independent techniques (Demir, 2020; Dertli & Çon, 2017; Kesmen & Kacmaz, 2011; Nalbantoglu et al., 2014; Purutoğlu et al., 2020; Taş et al., 2012). Dertli and Çon (2017) identified 15 different bacterial species and 17 yeast and mold species from kefir grains by culture-independent method and *Lb. kefiranofaciens* was found to be the dominant kefir associated bacterial strain. On the other hand, Purutoğlu et al*.* (2020) identified lactic acid bacteria and yeast from kefir grains by culture-dependent method and they have revealed that *Lactococcus lactis* strains were dominant among them. Moreover, Kesmen and Kacmaz (2011) determined *Lactococcus lactis* , *Leuconostoc mesenteroides* and *Lb. kefiri* as dominant strains in kefir grains analysed in culture-dependent method, while *Lb. kefiranofaciens* was dominant strain in culture-independent method.

It is possible to continue giving further examples, however, the message is already very clear; Considering the antiviral mechanisms, namely immunomodulation, antiviral metabolites, peptides, adherence and others, traditional fermented foods are surely more likely to provide health benefits due to their high microbial diversity, as compared to the commercial industrial products which are produced by a limited number of well-defined probiotic or starter cultures. Although it may not be possible to use the word 'probiotic' without providing proof by clinical trials, a high microbiodiversity, specifically of Lactobacilli increase the likelihood of providing several health benefits. These comparisons await further experimental analysis.

Conclusion

Both probiotic cultures and their metabolites in fermented foods have been proven to have antiviral activity both against upper respiratory tract and gastrointestinal viral infections. Among the antiviral mechanisms, most important ones appear to be their capability of modulating immune response, the production of antiviral metabolites and direct interaction of cells and the virus particles. Probiotics, when orally administered or introduced by nasal spraying, have been shown to thrive in the mouth and the nasopharynx and tonsils up to several days. This suggests, consumption of food is advantageous by initiating antiviral action from the beginning of the GI. Not only viable cells but non-viable probiotic cells (paraprobiotics) have also been shown to have health benefits, including antiviral action. Paraprobiotics provide a safer alternative to the administration of live probiotics at high numbers. Such dead cells naturally occur in fermented foods, especially upon long periods of storage and ripening. By being a natural source of paraprobiotics, diverse microbial species, mostly Lactobacilli, and rich metabolites, traditional fermented food products may be the hidden treasure of biological health benefits, including antiviral activity but this yet to be supported by experimental evidences.

Future Trends and Perspectives

There have been a vast number of publications up to date on the health benefits of probiotic lactic acid bacteria (LAB), including their antiviral activities, however, these mechanisms are far from being fully understood. The complex interrelationship of multiple mechanisms arising from the presence of single or combinations of LAB, viable or dead cells, consumption of LAB in the form of tablets or as part of fermented foods etc., influences the final cross-talk of bacterial and host cells, as well as bacterial and viral interactions and the presence/absence and action of viral antagonistic chemicals. The enormous variety of viruses both respiratory and gastrointestinal makes the whole story even more complicated. The complex interplay of these biological and biochemical components requires further dedicated research for a clearer understanding of these antiviral mechanisms. In the literature, *in situ* studies with the antiviral properties of fermented food metabolites, including peptides are inadequate and should also be considered in future studies. Besides all aforementioned viruses in the previous studies, the antiviral effects of peptides on Covid-19, which is the most troublesome virus of today, can be examined. Finally, clinical tests analyzing the antiviral and other health benefits of traditional food products with a rich

variety of LAB are few, if any, in the literature, and should be of concern in future studies.

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BIOTECH STUDIES

RESEARCH PAPER

Short-term inhibitory effects of TiO_2 NPs on Anammox process

Tuğba Sarı[1], Ayşe Özlem Yar-Bektaş[2], Deniz Akgül[2]*

[1]Department of Bioengineering, Marmara University, 34722 Göztepe, Istanbul, Turkey.
[2]Department of Environmental Engineering, Marmara University, 34722 Göztepe, Istanbul, Turkey.

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***Corresponding Author**
Tel.: +90 536 308 6006
E-mail:
deniz.tufan@marmara.edu.tr

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Abstract

Anammox process has brought about cost-effective, eco-friendly, and innovative technologies to wastewater treatment by reducing the operational cost of treatment plants and decreasing greenhouse gas emissions. Titanium dioxide (TiO_2), as one of the most prevalent nanoparticles (NPs) in the world, is being used in various consumer products and applications. In recent years, studies have focused on potential toxicological impacts of NPs on biological processes due to their endless production and consumption. In this context, the first time in the literature, 24 h acute TiO_2 NPs exposure on Anammox process was investigated. Deterioration on anammox activity gradually increased with increasing applied TiO_2 NPs concentration. At 300 mg/L exposure dose, nitrogen removal rate dramatically decreased to 37.09 ± 0.24 mgN/gVSS.d and a severe inhibition (80.57% ± 1.17%) was observed. Among the several curve fit models, non-linear second order polynomial (quadratic) model was the best fit one with IC_{50} of 154 mg/L. Scanning electron microscope (SEM) images demonstrated the tendency of TiO_2 NPs to aggregate and attach to the surface of the bacteria. Extracellular polymeric substance (EPS) response of anammox bacteria was also investigated and it was found that, the total EPS content gradually decreased by increasing TiO_2 NPs concentration.

Introduction

Nitrogen participates in the structure of many important biomolecules such as ATP, chlorophyll, nucleic acids and vitamins as the most abundant element in the universe. However, nitrogen in dinitrogen gas (N_2) form is inaccessible to most of the living organisms, especially primary producers. Therefore, the nitrogen cycle can be thought as one of the most essential biological processes that ensure the continuity of life (Stein & Klotz, 2016). Nitrification and denitrification are two essential processes involved in the nitrogen cycle. Inspired by the uniqueness of the nature, they have been immensely applied to wastewater treatment systems in order to remove nitrogen and prevent eutrophication. In these processes, ammonia (NH_3) is firstly transformed to nitrite (NO_2^-) and subsequently to nitrate (NO_3^-) by nitrifiers. Thereafter, NO_3^- is reduced into various nitrogen forms including NO_2^-, nitric oxide (NO), nitrous oxide (N_2O) and N_2 by denitrifiers (Heil et al., 2016; Robertson & Groffman, 2015).

Towards the end of the 20th century, global nitrogen cycle has been updated along with the discovery of anaerobic ammonium oxidation (anammox) bacteria by Mulder et al. (1995). Anammox reaction refers to the direct conversion of ammonium to N_2 via nitrite, in the absence of oxygen. Furthermore, Anammox process has brought about cost-effective, eco-friendly and innovative technologies to environmental fields e.g. industrial and domestic wastewater treatment systems (Peeters & van Niftrik, 2019). Remarkable reduction of operational cost is the main advantage of this promising technology over conventional nitrification/denitrification. In this process, there is no need for external carbon source, sludge production is 80% less, and aeration cost decrease by 60% (Cao et al., 2017).

Despite all advantages of the Anammox process and its application in over 200 wastewater treatment

facilities worldwide, anammox bacteria have slow growth rate and low cell yield (Zhang et al., 2016) which make the start-up and operation challenging. They are also susceptible to both inhibitory compounds such as antibiotics, heavy metals, nanoparticles (NPs) (Jin et al., 2012), and environmental factors such as pH, temperature and dissolved oxygen. All the aforementioned factors restrict the application and industrialization of anammox-based processes.

The manipulation of physicochemical properties of NPs (1-100 nm) such as size, shape, charge and coating promotes the widespread implementation of them into various industries including medical, food, military and cosmetic sectors (Bhushan, 2017; Kaphle et al., 2018). The global nanomaterials market was valued USD 7.3 billion in 2016 and is expected to the reach USD 16.8 billion by 2022 (Liu et al., 2019). Titanium dioxide (TiO_2) as one of the most prevalent NPs in the world, is being used in many cosmetic products including sunscreens, beauty products, toothpastes. Dietary supplements, candies, sweets, prosthetic implants, food colorants, paints, plastics, pigment production, anti-fogging car mirrors, ink, coating, photocatalysts are other examples of TiO_2 NPs applications (Chen & Chen, 2017; Mohamed, 2018).

NPs may be released into the ecosystems at every stage of their life cycles. Not only the fate of them in the environment, but also their toxicity levels are determined by their physicochemical properties which undergo some changes in different environment matrices (Liu et al., 2019; Senapati & Kumar, 2018). A significant amount of NPs has been detected in wastewaters and solid waste landfill sites (Keller & Lazareva, 2014; Musee et al., 2011). Therefore, their potential environmental toxicity has become a great concern because of the continuous increment in manufacturing and utilization of NPs (Chen & Chen, 2017). Besides, considering their relatively small size, more freely movement and larger surface area, they can be more toxic than larger particles in the bulk (Gupta & Xie, 2018). This is because entrance of them into the cells is much easier and they can cause cell damage (Liu et al., 2011).

Previously, several studies have been published to reveal the potential impacts of NPs on biological wastewater treatment systems including biological nitrogen and phosphorus removal (Wu et al., 2018; Zheng et al., 2011), anaerobic digestion (Lombi et al., 2012; Mu & Chen, 2011; Zhang et al., 2019) and anammox process (Li et al., 2018; Xu et al., 2019; Z. Z. Zhang et al., 2018a). Currently, there are only 3 studies focusing on the potential impacts of TiO_2 NPs on anammox process. However, 2 of them highlight the chronic (long term) effects of TiO_2 NPs (X. J. Zhang et al., 2018b; Z. Z. Zhang et al., 2018b) while the last one implies the short-term effects of TiO_2 NPs on anammox with the acute exposure tests of 8 h (X. J. Zhang et al., 2018a). A recent study stated that, 12 h exposure of inhibitory compounds is not sufficient to observe the defense mechanisms of anammox bacteria due to their slow growth rate (Song et al., 2018). Therefore, although the studies published in the current literature have enhanced our knowledge about the potential impacts of TiO_2 NPs on anammox process, short-term inhibitory effects of TiO_2 NPs on this process has not clearly been demonstrated yet. In the light of them, this study aimed to investigate the acute impacts of TiO_2 NPs on anammox process. In order to achieve this, seven different TiO_2 NPs doses (1, 10, 50, 75, 100, 200, 300 mg/L) applied to laboratory-scale anammox bioreactor for 24 h. IC_{50} value of TiO_2 NPs was studied with several inhibition models. In addition, extracellular polymeric substance (EPS) response of enriched anammox culture and changes in surface morphology of anammox bacteria were revealed by SEM analysis.

Materials and Methods

Anammox seeding sludge and experimental setup

A 2L lab-scale anammox bioreactor was established in order to enrich the anammox bacteria and use for acute exposure tests. Experimental setup is illustrated in Figure 1. Seeding sludge was obtained from an up-flow anammox bioreactor which have been being operated for more than 10 years in our laboratory. The reactor was operated in sequencing batch reactor (SBR)

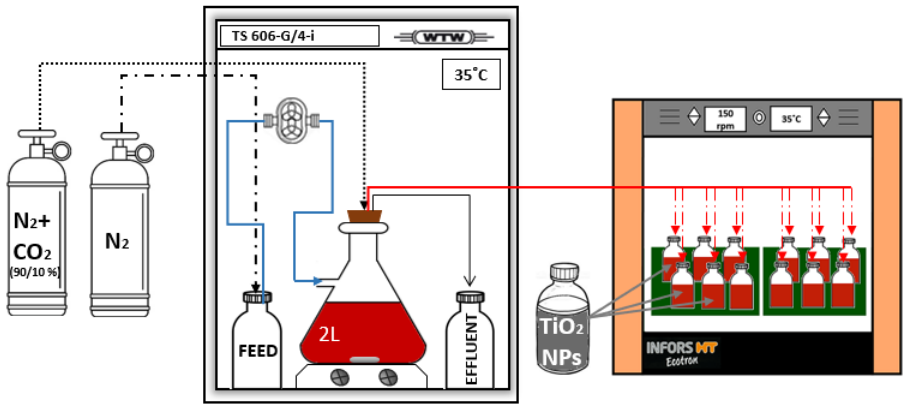

Figure 1. Experimental setup of the study.

mode for 24 h cycle with fill, reaction, settling and effluent withdrawal time of 20 min, 22.67 h, 40 min and 20 min, respectively. Synthetic wastewater to feed the Anammox bioreactor was prepared as previously described by Yapsakli et al. (2017), containing 1:1.15 ratio of NH_4^+-N and NO_2^--N, 0.073 g/L $CaCl_2$, 0.174 g/L K_2HPO_4, 0.102 g/L $MgCl_2$, 1 ml of trace element solution 1 (10 g/L $Na_2EDTA{\cdot}2H_2O$ and 5 g/L $FeSO_4$) and trace element solution 2 (10 g/L $Na_2EDTA{\cdot}2H_2O$, 0.43 g/L $ZnSO_4{\cdot}7H_2O$, 0.24 g/L $CoCl_2{\cdot}6H_2O$, 0.99 g/L $MnCl_2{\cdot}4H_2O$, 0.25 g/L $CuSO_4{\cdot}5H_2O$, 0.19 g/L $NiCl_2{\cdot}6H_2O$, and 0.014 g/L H_3BO_4). Besides, in order to prevent anaerobic environment that may lead to septic conditions, 50 mg/L $NaNO_3$ was introduced to the synthetic feed solution. 1.04 g/L $NaHCO_3$ was also supplied to the synthetic feed to prevent pH changes during the operation. Prior to feeding, in order to get rid of oxygen suppression risk, dissolved oxygen was stripped from the synthetic wastewater solution with N_2 gas.

A TS 606-G/4-i incubator (WTW, Germany) was used to maintain the mesophilic environment (35 ± 0.5 ˚C) to the anammox bioreactor. Hydraulic retention time (HRT) and pH were set to be 2 days and 7.5 ± 0.3, respectively. N_2/CO_2 (90/10%) gas mixture was also supplied to the reactor for inorganic carbon requirement.

Preparation of TiO_2 stock solution

The stock solution containing NPs, was prepared according to Mu et al. (2012). Commercially produced TiO_2 NPs (<25 nm) (Sigma Aldrich, USA) was added to the 0.1 mM sodium dodecylbenzene sulfonate (SDBS) to prepare 1 g/L TiO_2 NPs stock solution in order to provide stability of NPs and prevent agglomeration of them. Thereafter, stock solution was sonicated for 1 h at 25 °C, 40 kHz, 250 W by Sonopuls Ultrasonic Homogenizer (Bandelin, Germany).

Acute exposure tests

In order to conduct batch exposure tests, synthetic wastewater solution, NPs and enriched anammox culture were transferred into amber serum flasks having an effective volume of 50 mL. Synthetic wastewater composition (100 mg/L NH_4^+-N and 115 mg/L NO_2^--N) was the same with that of parent reactor and pH was adjusted to 7.5 ± 0.2. Each serum flask contained 1.5 g/L ± 0.5 g/L volatile suspended solids (VSS). Prior to starting experiment, serum flasks were purged by N_2 gas for 3 min to remove dissolved oxygen. Subsequently, all serum flasks were sealed with rubber stoppers and aluminum crimps. 24 h incubation was performed in Ecotron incubation shaker (INFORS HT, Sweden) at 35 °C and 150 rpm. In order to determine the specific nitrogen removal rates, 0.5 mL well-mixed liquid samples were taken from the flasks every 3 hours. Each exposure experiment was conducted in triplicate. Four different inhibition models were applied by GraphPad Prism (version 7.03) software package to determine the best fit model and estimate the IC_{50} value. Equations of inhibition models are listed below.

Linear regression

$$I\% = m \times NPs + n \quad (1)$$

Modified non-competitive inhibition model

$$I\% = 100 \times \left(1 - \frac{1}{1+\left(\frac{NPs}{a}\right)^b}\right) \quad (2)$$

Non-linear dose-response inhibition models

Inhibitor vs. normalized response

$$A\% = \frac{100}{1+\frac{NPs}{a}} \quad (3)$$

Inhibitor vs. normalized response -- Variable slope

$$A\% = \frac{100}{1+\left(\frac{a}{NPs}\right)^{Hill\ slope}} \quad (4)$$

Non-linear second order polynomial (quadratic) model

$$I\% = B0 + B1 \times NPs + B2 \times NPs^2 \quad (5)$$

Where A% and I% represent the activity and inhibition response of anammox process, respectively, NPs is the applied nanoparticle concentration, a is the value causing 50% inhibition on nitrogen removal rate, b is a fitting parameter and m, B0, B1, B2 are coefficients.

Analytical procedures

All analytical procedures including VSS, total suspended solids (TSS), NH_4^+-N and NO_2^--N were determined according to Standard Methods (APHA, 2005). pH was measured by HQ40D digital portable multimeter (HACH, USA).

EPS analysis

Protein (PN) and polysaccharides (PS) contents account for 75-89% of total EPS content (Tsuneda et al., 2003). Therefore, PN and PS concentrations in the samples were determined by modified Lowry method and Anthrone method, respectively. Prior to EPS quantification, modified heat extraction method, which was previously described by Morgan et al. (1990), was performed to extract EPS from mixed-liquor sludge samples. Subsequently, supernatant of the samples was filtered through 0.45 µM and stored at -20 °C. Each measurement was performed with two independent samples and each sample was tested in duplicate. A UV-2450 Spectrophotometer (Shimadzu, Japan) was used to conduct EPS measurements.

SEM analysis

Mixed-liquor sludge samples were firstly centrifuged at 3000 rpm for 10 min. Following the removal of supernatant, the pellet parts were washed three times for 3 min by 0.1 M phosphate buffer to get

rid of the unbounded materials. Thereafter, the pellets were fixed by fixation solution containing 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer solution (PBS) at 4˚C for 4 h. In order to remove fixation solution, washing procedure was applied again. Finally, the pellets were dehydrated in 50%, 70%, 80%, 90% and 100% ethanol solutions, respectively for 15 min and transferred on grids. Following air drying, the samples were coated with platinum and the images were obtained by environmental scanning electron microscope (Philips XL30 ESEM-FEG /EDAX).

Statistical analysis

The results of the experiments were represented as the mean ± standard deviation using Microsoft Excel. Paired t-test was also performed considering $P < 0.05$ as statistically significant.

Results and Discussion

Acute exposure of TiO_2 NPs on nitrogen removal performance of anammox process

SDBS, which is known as an anionic surfactant, was used as a dispersing agent in order to enhance the NPs stability and prevent their aggregation in the stock NPs solution. Previously published studies revealed no negative impacts of SDBS at low concentrations on biological processes including anaerobic treatment and anammox systems (Mu & Chen, 2011; Mu et al., 2012; Qiao et al., 2016; Zhang et al., 2017). In the current study, it was observed that, 0.1 mM SDBS has no statistically significant inhibitory impact on nitrogen removal efficiency of Anammox process (α=0.05, $P = 0.25 > 0.05$ for μ1=μ2) (Figure 2).

In the scope of exposure tests, anammox bacteria were exposed to seven different TiO_2 NPs dosages from 1 mg/L to 300 mg/L for 24 h incubation in order to observe the acute effects of TiO_2 NPs on nitrogen removal performance. Nitrogen concentrations in amber serum flasks were analyzed at every 3 h.

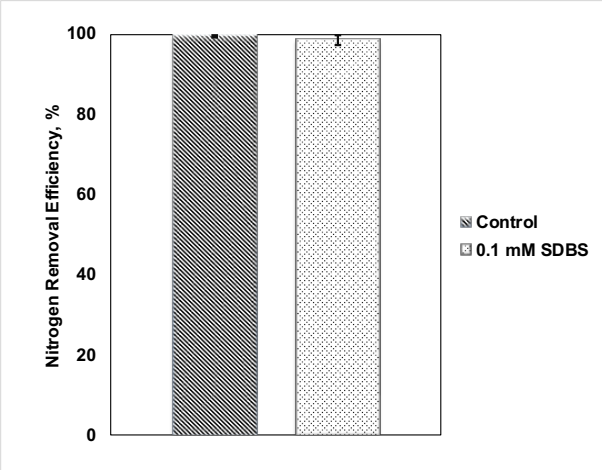

Figure 2. The effect of 0.1 mM SDBS on nitrogen removal efficiency. Data indicate average, and error bars represent standard deviations of the results from three independent sampling.

Thereafter, nitrogen removal rates (NRR) of the enriched anammox culture were calculated for each dose using the maximum slope of nitrogen removal vs time curves. In the absence of TiO_2 NPs (0 mg/L), NRR of anammox system was detected to be 190.94 ± 12.93 mgN/ gVSS.d. During the acute exposures of relatively small TiO_2 NPs dosages (1 mg/L and 10 mg/L), NRR was not significantly affected (α=0.05, $P > 0.05$ for μ1=μ2) (Figure 3a). As the applied NPs dosage increased, the inhibition percentage also increased (Figure 3b). Along with the increase of NPs dose to 50 mg/L, percentage inhibition reached to 20.62% ± 1.70% and a statistically significant decrease was observed in NRR (α=0.05, $P = 0.02 < 0.05$ for μ1=μ2). When the applied dose was risen to 100 mg/L and/or higher loads, extremely significant deteriorations were observed in NRR (α=0.05, $P < 0.01$ for μ1=μ2). In the presence of 300 mg/L TiO_2 NPs dosage, NRR was dramatically decreased to 37.09 ± 0.24 mgN/ gVSS.d and a severe inhibition (80.57 ± 1.17%) was observed (Figure 3a & 3b).

In order to examine the response of anammox bacteria to NPs stress, several inhibition models namely,

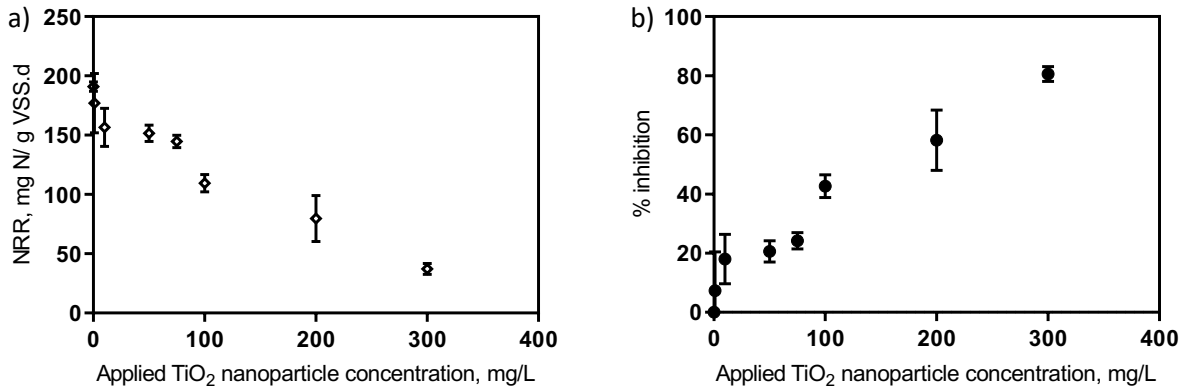

Figure 3. Acute effects of TiO_2 NPs on anammox activity. a) nitrogen removal rate in the presence of TiO_2 NPs b) percent inhibition (%) response of the applied TiO_2 NPs dosage. Data indicate average, and error bars represent standard deviations of samples measured in triplicate.

linear regression, modified non-competitive inhibition model, non-linear dose-response inhibition models, and non-linear second order polynomial (quadratic) model were tested (Figure 4). In all models, the IC_{50} of TiO_2 NPs on anammox activity was calculated. Compliance of the experimental data and determining the reliable curve fitting were evaluated by the R^2 value. The best fit curve was obtained with the non-linear second order polynomial (quadratic) model. In this model, the IC_{50} was determined to be 154 mg/L (R^2= 0.964).

In the literature, there is only one study that highlights the short-term effects of TiO_2 NPs on Anammox process (X. J. Zhang et al., 2018a) with an exposure time of 8 h. In that study, it is reported that, 1 mg/L NPs concentration enhanced the anammox activity while 5-20 mg/L dosages inhibited the anammox activity. It was also emphasized that 50 mg/L TiO_2 NPs dose showed lower suppression. On the contrary, this study demonstrated that acute exposure of TiO_2 NPs caused inhibition on Anammox process. The inhibition severity also rose as the applied dose was increased. The difference in the incubation period of acute exposure tests may be the most probable reason for different outcomes. In a recently published study, it was stated that, less than 12 h exposure period is not adequate to examine the anammox response against the inhibitory factors (Song et al., 2018). Besides these, not only the experimental setups but also the use of anammox granules/flocs and/or dominant anammox species in the enriched anammox culture may lead to the variations in the findings.

Attitudes of NPs in Anammox reactor

At the end of each acute exposure test, surface morphology of anammox bacteria was observed by SEM images. TiO_2 NPs mainly exist in solid phases because of their highly insoluble characteristic (Zheng et al., 2011). They tend to adsorb on biological surfaces or form aggregates with each other rather than the ion form (Cervantes-Aviles et al., 2017; Kokalj et al., 2018). As shown in Figure 5, TiO_2 NPs made clusters and attached to the surface of the bacteria. Moreover, when the applied dosage increased, amount of attached NPs also increased. Therefore, the substrate transfer rate between the environment and bacteria was possibly eliminated and NRR of Anammox process decreased. On the other hand, it has already been known that the larger the particle size is, the lower the toxicity. Hence,

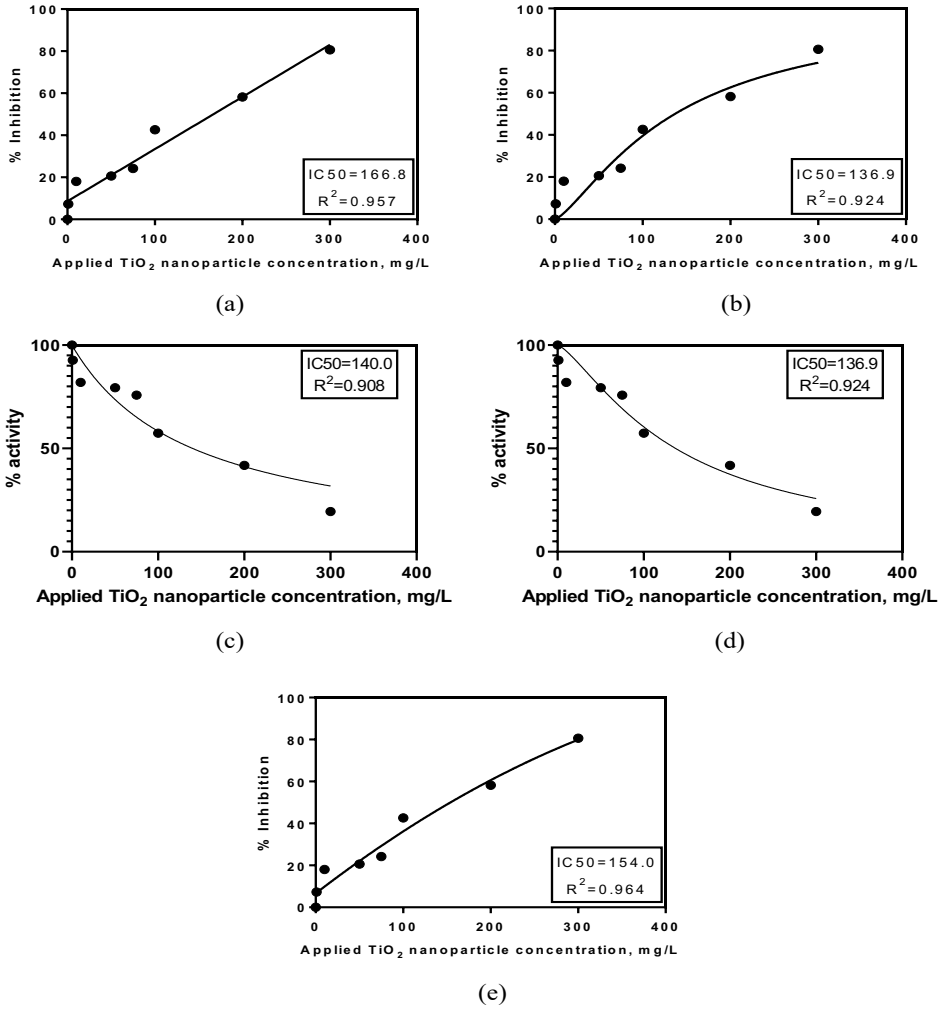

Figure 4. Inhibition response of the applied TiO_2 NPs dosage with different inhibition models. a) linear regression; b) modified non-competitive model; c) Inhibitor vs. normalized response; d) Inhibitor vs. normalized response - Variable slope; e) non-linear second order polynomial (quadratic) model.

due to the clustering of TiO_2 NPs, particle size may increase, which may lead to lower toxicity. As a different perspective, the EPS produced by the bacteria may trap the NPs and protect the bacteria assisting the aggregation of NPs. Even though, it was formerly speculated that TiO_2 NPs cannot be accumulated in high quantities on not only bacterial cell wall but also EPS due to electrostatic repulsions (Huangfu et al., 2019), SEM analysis exhibited the aggregation of TiO_2 NPs on anammox bacteria. A previous study also demonstrated the deposition of significant amount of metal oxide NPs including TiO_2 on the outer layer of anammox granules (Z. Z. Zhang et al., 2018b).

EPS response of Anammox bacteria

EPS, an organic matrix containing proteins, carbohydrates, DNA and lipids (Tang et al., 2018), is secreted by various microorganisms in anammox granules. Not only it protects microorganisms from adverse conditions as a first barrier against to environmental stress such as heavy metals and NPs, but also it plays an important role in accelerating the granulation process, improving stability of the matrix structure and granules (Yang et al., 2013; Zhang et al., 2015, 2017). Therefore, it is necessary to track EPS released by enriched anammox culture after acute exposure tests in order to understand the role of EPS in the resistance of anammox system against TiO_2 NPs.

Type of biological systems and the properties of NPs specify the potential effects of metal NPs (Xu et al., 2019). Thus, there have been various outcomes about the EPS responses of microorganisms. Decrement in PN content of anaerobic granular sludge against elevating ZnO NP concentration from 10 to 200 mg/g TSS was reported by Mu et al. (2012) while some studies were stated increment in EPS secretion in the existence of metal NPs including CuO, CeO_2 (Hou et al., 2015; Ma et al., 2013; You et al., 2017). Furthermore, some studies revealed that, NPs (ZnO, NiO) at lower concentrations firstly could rise up the EPS amount while higher loads of the same NPs decrease the EPS amount (He et al., 2017; Xu et al., 2019).

In the current study, the total EPS content gradually decreased with escalating burden of TiO_2 NPs

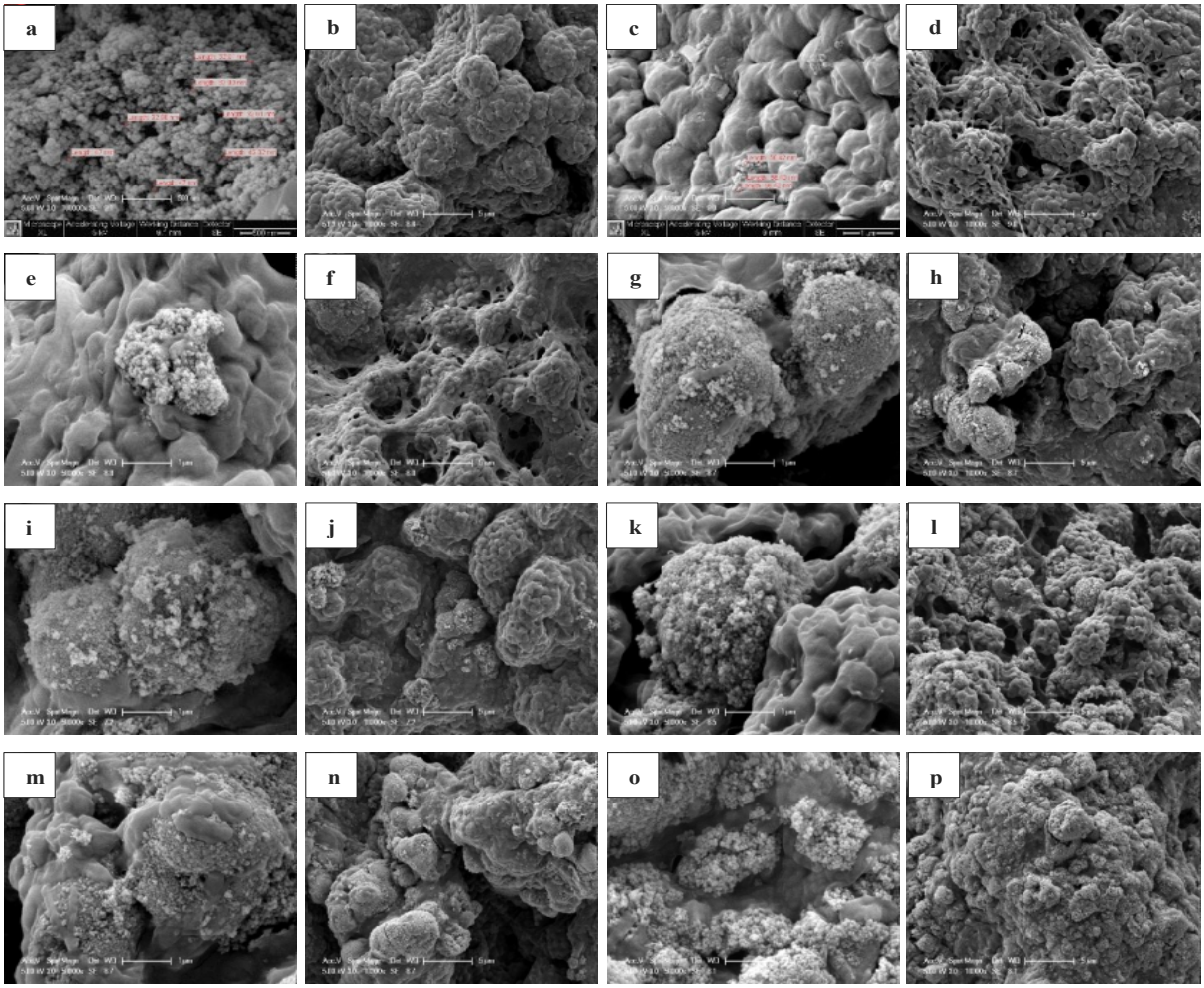

Figure 5. SEM images of anammox sludges after acute exposure tests a) TiO_2 NPs stock solution; b) anammox sludge; c) and d) 1 mg/L TiO_2 NPs exposure; e) and f) 10 mg/L TiO_2 NPs exposure; g) and h) 50 mg/L TiO_2 NPs exposure; i) and j) 75 mg/L TiO_2 NPs exposure; k) and l) 100 mg/L TiO_2 NPs exposure; m) and n) 200 mg/L TiO_2 NPs exposure; o) and p) 300 mg/L TiO_2 NPs exposure.

dosage (Figure 6). Numerically, 49.73% and 92.19% decrements in total EPS content was determined after the acute exposures of 50 mg/L and 300 mg/L TiO_2 NPs dosages, respectively. During all the exposure experiments, PN production was greater than PS production. Therefore, it could be interpreted that, sensitivity of PN to TiO_2 NPs was more than that of PS. Similar findings also revealed by other studies focused on other NPs (Xu et al., 2019; Xu et al., 2018).

In a recent study, Z. Z. Zhang et al. (2018b) investigated the long-term effects of TiO_2 NPs on Anammox process at three different NPs dosages (1 mg/L, 50 mg/L, 150 mg/L) for a month each and reported the enhancement of EPS production during the exposure period and highlighted the EPS secretion as adaptation strategy. In this study, however, the anammox biomass was exposed to shock loads of NPs within a short time. Hence, anammox bacteria may not be able to adapt to the changing environment and could not produce enough EPS to protect the cellular structure against the inhibitory compound. This phenomenon may also be explained by the longer lag phase of EPS secretion in anammox population compared to the heterotrophs because of their relatively slow growth rates (Song et al., 2018). Besides, the differences in exposure periods of the same NPs may change the EPS responses. Zhao et al. (2019) pointed out 24 h short-term exposure of ZnO NPs (5, 50, 150 mg/L) caused gradually decrement in EPS release by anammox granules. However, Sari et al. (2020) emphasized that, long-term exposure of ZnO NPs increased the EPS secretion in anammox reactor up to 40 mg/L NPs dosage. Consequently, in the current study, the EPS production gradually decreased inversely proportional to the increase in inhibition percentage.

Conclusion

Acute response of TiO_2 NPs on Anammox process at seven different dosages (1, 10, 50, 75, 100, 200, 300 mg/L) was examined for 24 h incubation period. When the concentration was risen to 50 mg/L, a significant deterioration on NRR was obtained. Inhibition percentage on anammox activity was gradually increased by escalating the burden of applied TiO_2 NPs dosage. At 300 mg/L exposure dose, NRR was dramatically decreased from 190.94 ± 12.93 to 37.09 ± 0.24 mgN/ gVSS.d and a severe inhibition (80.57% ± 1.17%) was observed. Several inhibition models were tested in order to estimate the IC_{50} value. Non-linear second order polynomial (quadratic) model was the best fit model with $R^2 = 0.964$. The IC_{50} value was determined to be 154 mg/L with this model. At the end of the acute exposure tests, surface morphology of anammox bacteria was also observed by SEM images. The results revealed that, TiO_2 NPs tended to form cluster and attached to the surface of the bacteria. Moreover, as the applied NPs dosage increased, amount of attached NPs also increased. Finally, EPS secretion by enriched anammox culture was tracked in order to analyze the response of the anammox system. The total EPS content gradually decreased by increasing TiO_2 NPs dosage which is inversely proportional to the increase in inhibition percentage of anammox activity.

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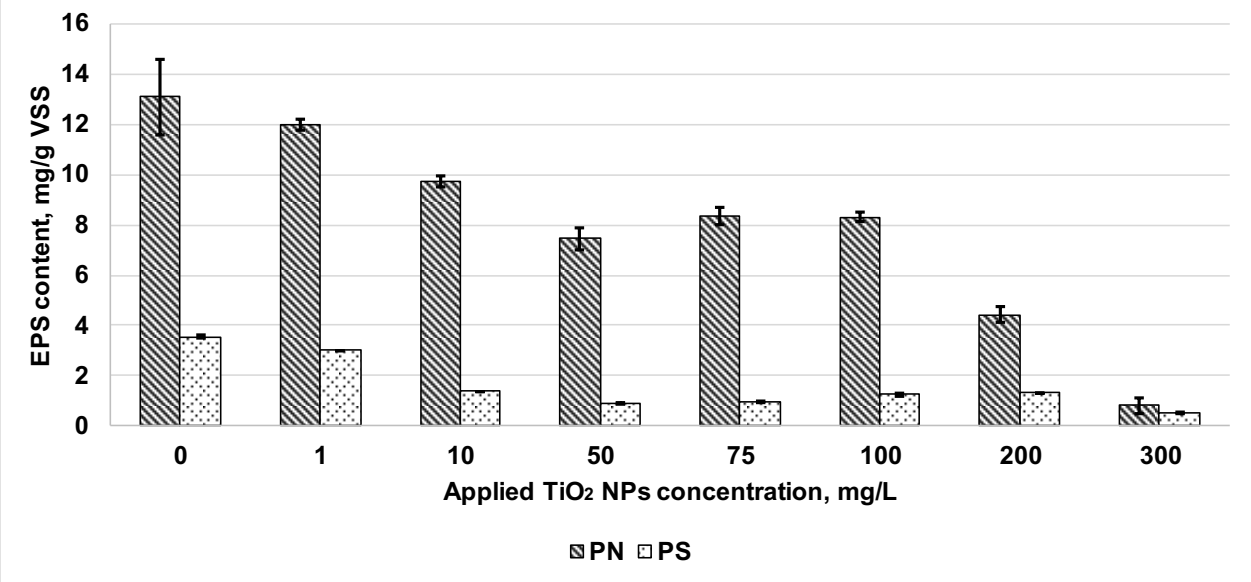

Figure 6. EPS secretion by enriched anammox culture exposed to TiO_2 NPs. Data indicate average, and error bars represent standard deviation of the results from two independent sampling, each tested in duplicate.

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BIOTECH STUDIES

RESEARCH PAPER

Genetic diversity analysis of some species in Brassicaceae family with ISSR markers

Fatma Gül Maraş-Vanlıoğlu[1,*], Hümeyra Yaman[1], Fatma Kayaçetin[2]

[1]Biotechnology Research Center, Field Crops Central Research Institute, Republic of Turkey Ministry of Agriculture and Forestry, 06170 Yenimahalle, Ankara, Turkey.
[2]Department of Breeding and Genetics, Field Crops Central Research Institute, Republic of Turkey Ministry of Agriculture and Forestry, 06170 Yenimahalle, Ankara, Turkey.

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***Corresponding Author**
Tel.: +90 551 233 0330
E-mail:
fatmagul.maras@tarimorman.gov.tr

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Abstract

Brassicaceae is one of the biggest family which have thousands of species all around the world. In order to use wild mustard in a breeding process, their genetic kinship levels must be defined. Inter simple sequence repeats (ISSRs) are one of the common markers to evaluate genetic diversity. Here, 28 mustard genotypes representing four taxa, 17 of *Brassica juncea*, 2 of *B. nigra*, 2 of *B. rapa*, and 7 of *B. arvensis*, were investigated with seven ISSR primers. Totally, 160 bands were scored out of which 88.75% showed polymorphism. The polymorphism information content (PIC) varied from 0.25 to 0.40. The average heterozygosity (Hav), multiplex ratio (MR), marker index (MI), and resolving power (Rp) were calculated as 0.33, 9.07, 2.99, and 8.29, respectively. STRUCTURE (v. 2.3.4) analysis unraveled two subpopulations (K=2). The dendrogram, constructed based on Jaccard similarity coefficient using the Unweighted Pair Group Average (UPGMA), in which, the first branch consisted of *B. juncea*, *B. nigra* and *B. rapa*, and the second branch consisted of *B. arvensis,* supported the results of STRUCTURE analysis. Additionally, principal component analysis (PCA) analysis supported the dendrogram and clearly separated the four taxa. This study showed that ISSRs would be useful to determine the genetic diversity in the Brassicaceae family.

Introduction

In the world, there are about 372 genera and 4060 species of Brassicaceae family. Turkey has great biodiversity and the Irano-Turanian region is the possible origin district of this family (Karl & Koch, 2013). This region consists of approximately 900 species of Brassicaceae family, and Turkey has more than 606 species on its own and out of which 226 endemics (Koch et al., 2017; Mohammadin et al., 2017).

Investigation of Brassica genus was shown that it has a very long evolutionary process. The genus firstly took place in domestication as vegetables, and then as edible oilseed crops (Prakash et al., 2009; Kaur et al., 2014). It has three diploid species – *B. rapa* (2n=20, AA genome), *B. nigra* (2n=16, BB genome), and *B. oleracea* (2n=18, CC genome), and three amphidiploid species – *B. juncea* (2n=36, AABB genome), *B. carinata* (2n=34, BBCC genome), and *B. napus* (2n=38, AACC genome) (Jiang et al., 2015). The relationship among these species was explained a long time ago (U, 1935). Furthermore, *B. arvensis* (*Sinapis arvensis*) has a close chloroplast and nuclear genome homology with *B. nigra* (Pankin & Khavkin, 2011; Prakash et al., 2009). Each species has been undergone different domestication process for different purposes like root vegetables (turnip), green leaves (Chinese cabbage), floral parts (broccolini, cauliflower), or oilseeds (Baker et al., 2017).

In the world, the total production of Brassicas is 71.3 M tonnes and China is at the first rank with the 33.9 M tonnes in 2016. The second country is India with 8.7 M tonnes production and followed by Russia with 3.1 M tonnes production. Brassicas are also cultivated in Turkey and the production is about 790 kilotonnes (FAOSTAT, 2018). Additionally, the production of oilseed brassicas is at the second rank among vegetable oils

(Banuelos et al., 2013). These show how important to develop new varieties that will be adapted better and give a higher yield. To achieve that, the gene pool should be increased. For this purpose, wild mustard like *B. arvensis* should be included in breeding programs. For these wild species, in addition to the morphological observations and yield parameters, a molecular analysis should be done to determine the genetic relationship among breeding materials. After the discovery of molecular marker techniques, they have been used for genetic relationships among plants during crop improvement programs (Varshney et al., 2005). Thanks to genetic data obtained by molecular markers, not only developing new varieties could take a shorter time but also more accurate data could be obtained.

One of the molecular markers which is fast, affordable, highly discriminative, and confidential is inter simple sequence repeats (ISSRs) (Safari et al., 2013). These markers recognize short DNA fragment repeats (2-6 bp) throughout the whole genome. Since ISSR loci have a high polymorphism ratio, a lot of alleles can be observed (Moghaddam et al., 2009). Because of that ISSRs are ideal tools to determine the similarity and differences among genotypes (Abdelmigid, 2012). With the help of statistical analysis of ISSR markers, the similarity ratio among targeted species can easily be brought out.

ISSR markers have been used for genetic diversity analysis and molecular characterization of different plants like bread wheat (El-Sherbeny et al., 2020), taramira (Zafar-Pashanezhad et al., 2019), ginger (Baruah et al., 2019), kewda (Nasim et al., 2020), cassava (Afonso et al., 2019), flax (Ahmed et al., 2019), asparagus (Chen et al., 2020), and anise (Akçali-Giachino, 2020). Besides, ISSR markers were used for genetic diversity analysis and molecular characterization of *Brassica* by different researchers (Kalia et al., 2017; Kaur et al., 2014; Koch et al., 2017; Kong et al., 2011; Mohammadin et al., 2017; Takahashi et al., 2019; Verma et al., 2016).

In this study, 28 *Brassica* genotypes consist of four different taxa were investigated for the genetic diversity analysis through ISSR markers. It was aimed to determine the genetic relationship between wild and cultivated *Brassica* species for directing future breeding programs. This method was chosen since it is effective, highly polymorphic, affordable, and rapid.

Materials and Methods

Plant material

The plants used in this study were obtained from the Central Research Institute for Field Crops (CRIFIC) and U.S. Department of Agriculture - Agricultural Research Services (USDA-ARS). Twenty-eight genotypes were chosen to represent four Brassica taxa; 17 of *B. juncea*, 2 of *B. nigra*, 2 of *B*. *rapa*, and 7 of *B. arvensis*. All four taxa obtained were shown in Table 1. Young leaves of one to five plants were taken to represent each population and stored in silica gel.

Table 1. List of Brassica taxa and their locations.

No	Species	Locality
1	*Brassica juncea*	İzmir
2		Turkey
3		Tekirdağ
4		Tekirdağ
5		Kırklareli
6		Edirne
7		Tekirdağ
8		India
9		Pakistan
10		China
11		China
12		Russia
13		Russia
14		Germany
15		United States
16		India
17		Konya
18	*Brassica nigra*	Turkey
19		Turkey
20	*Brassica rapa*	Tekirdağ
21		Turkey
22	*Brassica arvensis*	Kırşehir
23		Tokat
24		Ankara
25		Tekirdağ
26		Şanlıurfa
27		Ankara
28		Ankara

DNA extraction and polymerase chain reaction

The DNA extraction of plants was made by using a DNeasy Plant Mini Kit from Qiagen (Hilden, Germany). The quality and quantity of samples were determined in the 1% agarose gel. PCR amplifications were done by following the instructions of the manufacturer (Jena Bioscience®) with some modifications; in a total volume of 20 µl master mix (5 x Red Load Taq Master); containing 20-50 ng of genomic DNA and 1µM primer. PCR reactions were started with an initial denaturation of 5 min at 94°C; followed by 35 cycles of 1 min at 94℃, 1 min at 52℃ (annealing) and 1.5 min at 72℃ (extension), with a final step at 72℃ for 7 min. PCR products were separated by 2.5% agarose gel electrophoresis with 1 x TBE buffer and stained with ethidium bromide. The gels were visualized by using VILBER Quantum-ST4 1100/26MX Imaging Cabinet (Vilber Smart Imaging, Marne-la-Vallée, France).

Data analysis of ISSR

Seven primers formed reliable polymorphic bands out of 78 ISSR primers were chosen for ISSR screening (Table 2). The GC ratio of the primers was between 44.4-56%.

Resolving power (Rp) of each ISSR primer was calculated as $Rp = \sum I_b$ in which the value of I_b (band informativeness) is determined as $1 - (2 \times |0.5 - p|)$ and p is the proportion of accessions containing the band (Prevost & Wilkinson, 1999).

The polymorphism information content (PIC) of an ISSR locus was calculated as $PIC = 2p \times (1 - p)$, where p is the proportion of the accessions with a band and $(1 - p)$ is the proportion of the accessions without a band (Roldan-Ruiz et al., 2000). Average heterozygosity (Hav) was calculated by taking the average of PIC values obtained for all the markers used in and the formula is as below:

$$Hav = \sum[2p \times (1 - p)] / N$$

Multiplex ratio (MR) was obtained by the multiplication of the mean value of fragments amplified by genotypes to a specific marker (n) with polymorphic band ratio (β) as declared by (Kumar et al., 2014; Powell et al., 1996).

$$MR = n \times \beta$$

Marker index (MI) was estimated by the multiplication of Hav with MR (Powell et al., 1996).

$$MI = Hav \times MR$$

According to the fragment sizes, bands were recorded as present (1) and absent (0). Clustering analysis (CA) and principal component analysis (PCA) were made by using NTSYSpc ver. 2.1. program of IBM PC (Rohlf, 2000). A Mantel test was applied to DICE, simple matching (SM), and Jaccard similarity coefficients (Mantel, 1967). Unweighted Pair Group Average (UPGMA) clustering was made by using Jaccard similarity coefficient for CA. PCA was obtained by applying the following steps: a distance matrix between the samples was calculated, the matrix of distances was double-centered, and finally, for the plot, the double-centered matrix was factored (Rohlf, 2000).

The population structure was investigated using STRUCTURE software (v. 2.3.4) that separates groups according to allelic frequencies (Pritchard et al., 2000). The hypothetical number of subpopulations (K) was set as a continuous series of K from 1 to 10 in 10 independent runs. A burn-in period of 100,000 steps was followed by 100,000 Monte Carlo Markov Chain (MCMC) iterations (Evanno et al., 2005). The highest ΔK values were calculated through STRUCTURE HARVESTER to unravel the final population structure (Earl & vonHoldt, 2012). The subpopulation number was obtained from the STRUCTURE program by using the K values. The *Fst* value for the differentiation of population and expected heterozygosity in the same cluster was calculated with the STRUCTURE.

Results

In this study, seven primers were chosen out of 78 ISSR oligonucleotide primers to find out the genetic relationship among 28 *Brassica* genotypes. Totally, 142 polymorphic, 5 monomorphic, and 13 unique bands were acquired. The bands which were common for more than 95% genotypes were called monomorphic. The highest number of polymorphic bands was derived from UBC 827 with 26 bands and the average frequency per primer was 20.29. The polymorphism ratio changed between 65.2-100% and the average was found as 88.75%. The highest polymorphism ratio was obtained from UBC 827 with 100%. The band length scored from primers ranged approximately from 120 base pairs (bp) to 2500bp. The PIC value was ranged between 0.25 and 0.4 (Table 2). The Rp value was between 5.36 and 14.57 for each primer and the mean value for Rp was calculated as 8.29. The average heterozygosity (Hav) obtained from all over the primers was 0.33 and the MR value was recorded as 9.07. Finally, the MI value which shows the efficiency of a marker was 2.99. Mantel test (Mantel, 1967) was performed to determine the 'goodness of fit' for the UPGMA dendrograms and the maximum r-value was obtained by Jaccard similarity coefficient as 0.98264.

Table 2. List of the information of ISSR primers used for the 28 accessions of Brassica taxa. R=A, G; Y=C, T.

Primers	Sequence 5'→3'	Tm (°C)	GC (%)	Size (bp) min–max	# of poly. bands	# of mono. bands	# of unique bands	Polymorphism rate (%)	PIC	Rp
UBC814	$(CT)_8A$	46	47	220-2.500	17	-	1	94.4	0.34	6.86
UBC826	$(AC)_8C$	55	52.9	120-1.200	21	1	-	95.5	0.36	7.86
UBC827	$(AC)_8G$	55	52.9	150-1.300	26	-	-	100	0.40	14.57
UBC830	$(TG)_8G$	55	52.9	120-1.350	23	2	2	85.2	0.32	7.36
UBC834	$(AG)_8YT$	49-52	44.4-50	190-1.000	15	1	1	88.2	0.32	5.36
UBC835	$(AG)_8YC$	50-54	50-56	140-1200	25	-	2	92.6	0.34	10.71
UBC845	$(CT)_8RG$	48-52	50-56	230-2000	15	1	7	65.2	0.25	5.36
Total					142	5	13			
Average					20.29			88.75	0.33	8.29

According to the scoring result, *B. juncea* (10) originated from China has the highest number and ratio of the polymorphic locus with a value of 87 and 61.3%, respectively. Otherwise, *B. rapa* (21) originated from Turkey gave the lowest number and ratio of the polymorphic locus with a value of 51 and 35.9%, respectively.

With respect to the dendrogram generated by Jaccard similarity coefficient (Figure 1), the samples from *Brassica* taxa were collected in two main groups (at 30% similarity level). The first group included *B. arvensis* accessions collected from the different regions of Turkey showed genetic similarity with the ratio of 48-56%. The second group consisted of 21 genotypes; 17 of *B. juncea*, 2 of *B. nigra*, and 2 of *B. rapa*. The genetic similarity ratio among this group ranged from 36.5% to 99%. The second group also had 2 subclusters. One of these subclusters consisted of 2 *B. rapa* accessions originated from Turkey, the other consisted of *B. juncea* and *B. nigra* samples. Moreover, *B. nigra* originated from Turkey, and *B. juncea* originated from a different region of the world that occurred in two separate clusters.

According to the clustering analysis, the similarity level of *B. juncea* (2) and *B. juncea* (3) is 99%. *B. juncea* (2) was obtained from USDA-ARS and only information about this accession was that the origin was Turkey. On the other hand, it is known that *B. juncea* (3) was collected from Tekirdağ, a city in the northwest of Turkey. This suggested that *B. juncea* (2) might have been collected from Tekirdağ. The second-high similarity level was observed between *B. juncea* (4) and *B. juncea* (5) with a value of 97%, which are from Tekirdağ and Kırklareli, respectively. The other sample in the same cluster was *B. juncea* (6) from Edirne. All of these accessions were located on the northwest side of Turkey between the Marmara and the western Black Sea. *B. juncea* (12) and *B. juncea* (13) originated from Russia were similar to each other at a 92% similarity level.

The total number of bands (TNB) was 121 and 113 out of seven ISSR primers in *B. juncea* and *B. arvensis* accessions, respectively. The average number of bands per primer is 17.29 (*B. juncea*) and 16.14 (*B. arvensis*). The maximum number of amplified products was obtained from UBC 827 for both *B. juncea* and *B. arvensis* with the number of 23 and 21, respectively. The minimum number of bands was obtained as 11 from UBC 845 for *B. juncea* and 14 from UBC 834 for *B. arvensis*. The resolving power (Rp) of the primers used in *B. juncea* was observed between 2.6 for both UBC 834 and UBC 845 and 8.1 for UBC 827. For the *B. arvensis*, Rp ranged from 4.9 for UBC 830 and 10 for UBC 827. The number of private bands (NPB) obtained with primers is 9 which is from 6 out of the 7 ISSR primers. The NPB observed in all accessions of *B. nigra* was 3 that was absent in all accessions of *B. juncea*, *B. rapa*, and *B. arvensis*. NPBs were observed in all accessions of *B. rapa* was 2 that was absent from the rest. Finally, there were 4 private bands in all accessions of *B. arvensis*. The PIC value was obtained between 0.14 and 0.26 for *B. juncea*, the mean was 0.18, while it was between 0.21 and 0.39 for *B. arvensis* and its mean was 0.31 (Table 3). Since the number of *B. nigra* and *B. rapa* accessions is low, specific PIC value for these species was not included.

To investigate more, 2- dimensional PCA was computed based on the ISSR band pattern using the J similarity coefficient. (Figure 2). The first three eigenvectors occurred 47.01% of the total variance (29.29% the first vector, 9.54% the second vector, and 8.18% the third vector). The results were in line with cluster analysis and formed 4 groups *as B. juncea*, *B. rapa*, *B. nigra*, and *B. arvensis*. *B. juncea* (16) and *B. juncea* (17) had a high dissimilarity ratio with respect to the rest of *B. juncea* accessions according to Jaccard

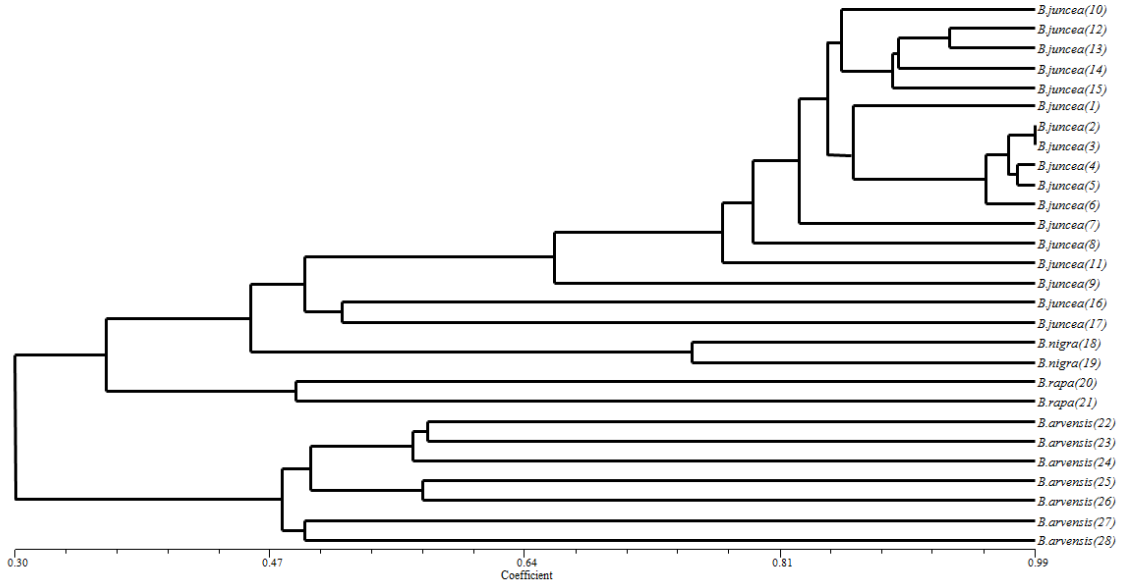

Figure 1. UPGMA-based cluster analysis of Brassicaea family with 28 populations.

similarity coefficient index and this situation was confirmed by PCA. As expected, similar taxa were placed closer to each other.

Table 3. List of the information of ISSR primers for *B. juncea* and *B. arvensis*. Total number of bands (TNB), resolving power (Rp), number of private bands (NPB), and polymorphism information content (PIC). R= A, G; Y=C, T.

Amphidiploid lines of *B. juncea* (2n=4x=36)					
Primer	**TNB**	**Polymorphism rate (%)**	***Rp***	**NPB**	***PIC***
UBC814	14	92.9	3.4	-	0.20
UBC826	22	77.3	4.1	-	0.15
UBC827	23	87	8.1	-	0.26
UBC830	21	61.9	3.9	-	0.14
UBC834	13	69.2	2.6	-	0.15
UBC835	17	88.2	4.4	-	0.19
UBC845	11	90.9	2.6	-	0.19
Total	121		29.1	-	
Average	17.3	80.2	4.2		0.18

Diploid lines of *B. arvensis* (2n=2x=18)					
Primer	**TNB**	**Polymorphism rate (%)**	***Rp***	**NPB**	***PIC***
UBC814	12	91.7	5.6	-	0.39
UBC826	17	76.5	6.9	1	0.28
UBC827	21	95.2	10	1	0.34
UBC830	16	62.5	4.6	1	0.21
UBC834	14	85.7	7.7	-	0.36
UBC835	17	70.6	6.9	1	0.26
UBC845	16	93.8	7.7	-	0.34
Total	113		49.4	4	
Average	16.1	82.3	7.1		0.31

STRUCTURE (v. 2.3.4) analysis was used to analyze population structure. K values for sub-populations were determined as between 1 – 10 and the peaks were detected at K = 2 (Figure 3b) according to the computational result of ΔK. At K = 2, the first cluster (Figure 3a, red) contains all *B. arvensis* accessions and the second cluster (Figure 3a, green) contains the 12 of 17 *B. juncea* accessions. The rest is in the transition between the two subpopulations (Figure 3a). These are compatible with the dendrogram (Figure 1) obtained from UPGMA method. The mean expected heterozygosity and *Fst* values were calculated as 0.2308 and 0.4212, respectively. These high values are the indicator of high heterozygosity among the genotypes.

Discussion

For breeding programs, obtaining of the targeted characters highly depends on having a big genetic pool. At this point, wild relatives of cultivated species have a great importance. These wild relatives can be used as a parent on the breeding process (Lara-Fioreze et al., 2013). Because of that, the main target of this study was to determine the genetic relationship between wild and cultivated *Brassica* species for directing future breeding programs.

There is a lot of research investigating the relationships in the Brassicaceae family by using microsatellite markers (El-Esawi et al., 2016; Singh et al., 2018; Thakur et al., 2017b). Also, there are a lot of studies in which specifically ISSR markers used to determine genetic relationships in the Brassicaceae family (Khalil & El-Zayat, 2019; Safari et al., 2013; Shen et al., 2016; Wang et al., 2017).

In this study using ISSR markers, the rate of polymorphism was obtained as 88.75% and the number of polymorphic bands per primer was 20.29. In another study conducted in *B. juncea*, polymorphism rate and the number of polymorphic bands per primer were 91.2% and 15.73, respectively (Gupta et al., 2014).

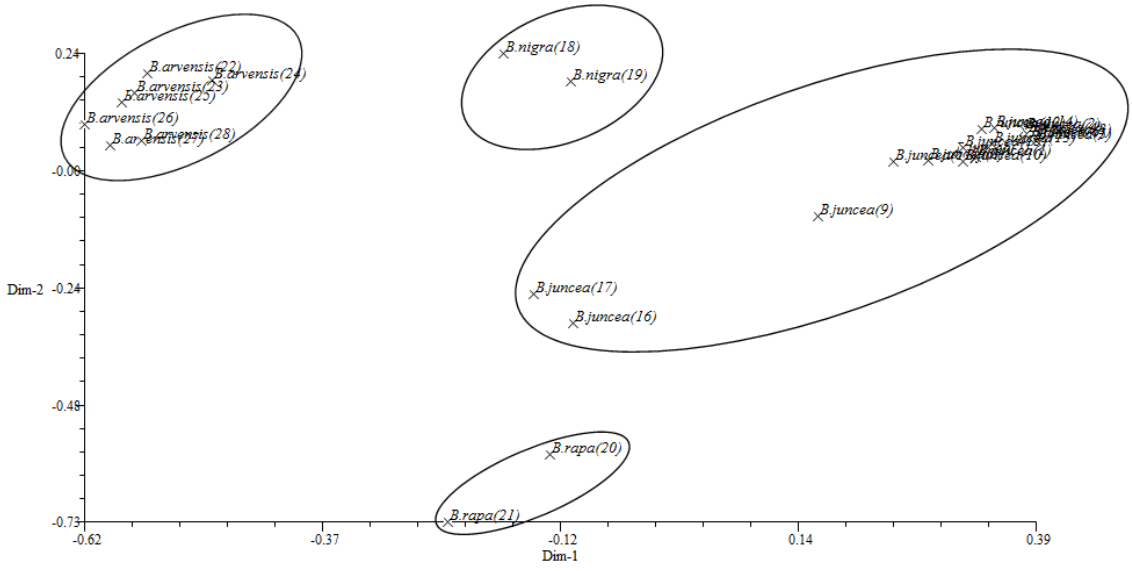

Figure 2. Two-dimensional PCA of 4 Brassica taxa with 28 accessions.

Furthermore, Abdelmigid et al. (2012) showed that the polymorphism rate was 87% and the number of polymorphic bands per primer was 13.4 in *B. napus*. The polymorphism rate of both studies was similar but the polymorphic bands per primer were higher in our study which could be because of different populations and markers.

Gohel and Mehta (2014) determined the genetic diversity among the 20 Indian mustard (*Brassica juncea*) genotypes grown in the northern states of India with ISSR primers. The dendrogram, which they drew according to Jaccard similarity index, was divided into two major branches and their similarities ranged from 47.8% to 100%. Here, we found that the similarity level among Indian origin *B. juncea* was determined as 50.6% and they divided into two different clusters. Similarly, Yadav and Rana worked with 30 Indian mustard genotypes in 2012, and found that the range of similarity was changing between 50% and 100%.

According to the triangle of U theory, *B. juncea* (2n = 36) is a hybrid of *B. nigra* (2n = 16) and *B. rapa* (2n = 20), which is closer to *B. nigra* in terms of genetic distance. Based on the results obtained in the current study, *B. nigra* was similar to *B. juncea* in the dendrogram. Similarly, in two different studies, it was found that *B. juncea* was closer to *B. nigra* more than *B. rapa* (Kaur et al., 2014; Thakur et al., 2017b).

For *B. nigra*, *B. rapa*, and *B. arvensis*, private ISSR bands were obtained. There are three, two, and four private bands for *B. nigra*, *B. rapa*, and *B. arvensis* accessions, respectively. These new markers could be used as a molecular tool to discriminate each species from the rest after testing these markers with more accessions from each species. A similar pattern had been described earlier to discriminate *Brachypodium distachyon* and *Brachypodium hybridum* species from each other (Contreras et al., 2017).

Additionally, a total of 13 unique bands were obtained from 5 of the 7 ISSR primers used in the study. These bands have great potential because they can be converted into Sequence Tagged Site (STS) or Sequence Characterized Amplified Regions (SCARs) (Gupta et al., 2014). Moreover, unique bands can be used to separate the cultivars from each other without the need for field trials (Fernández et al., 2002). In other words, unique bands obtained in this study can be used to obtain a genotype-specific profile. For example, in the current study, 5 of the 13 unique bands obtained from the ISSR marker screening belong to *B.rapa* (20), while 3 unique bands belong to *B.arvensis* (26) and 2 unique bands belong to *B.juncea* (9). Similarly, in a study conducted in 23 *B. juncea* accessions, originated from North India with 15 RAPD markers, 21 genetic specific unique bands

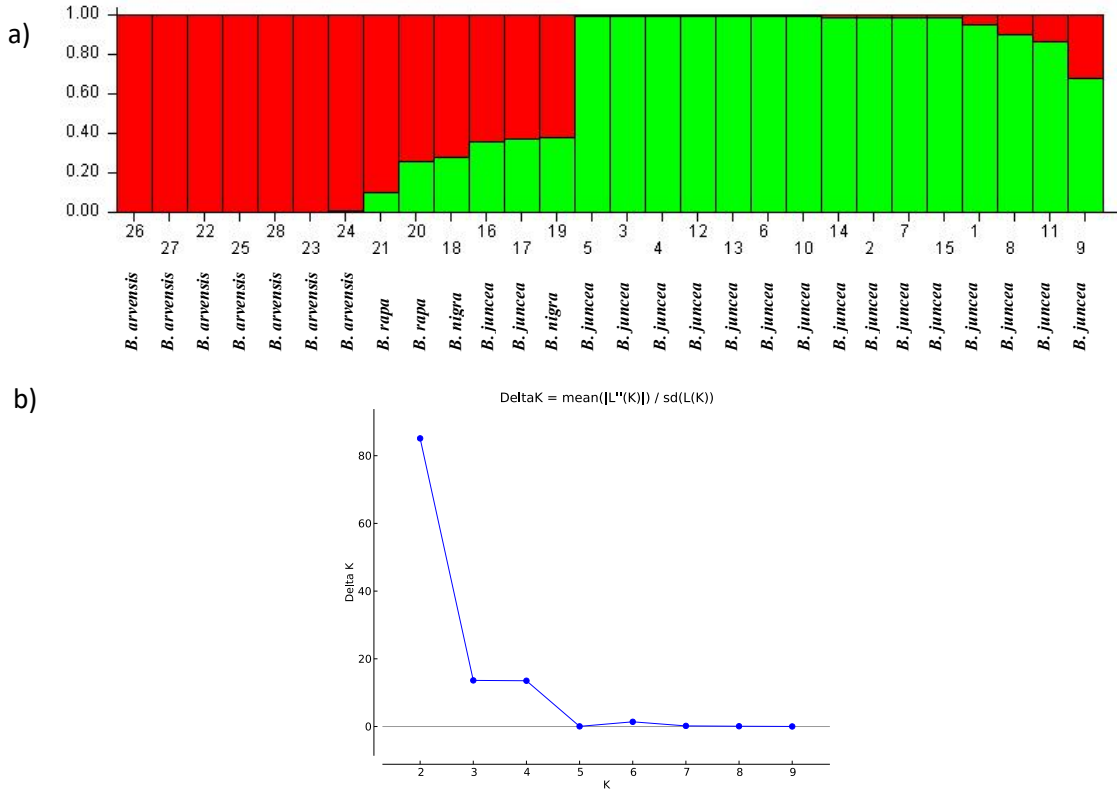

Figure 3. (a) Population structure of 28 Brassica genotypes using 160 loci based on ISSR scoring at K = 2. Each accession is indicated by a vertical line and each color represents a different cluster. (b) ΔK is computed between K = 1 – 10 and the peak value was obtained at K = 2.

were obtained from 12 RAPD markers (Gupta et al., 2014).

To understand the effectiveness of ISSR markers used, Hav, MR, MI, and Rp values were calculated. In this study, the PIC value was ranged between 0.25 and 0.4, and the Hav calculated from PIC value was 0.33 which is supported by Mahjoob et al. (2016). There are other studies for different plants like *Tribulus terrestris* (Sarwat et al., 2008), anise (Akçali-Giachino, 2020), and taramira (Zafar-Pashanezhad et al., 2019) and the value obtained for Hav are pretty much similar. The presence of higher MR value is always desirable for genetic diversity analysis. Here, we calculated MR value as 9.07 which was higher than the published other studies related to not only Brassicas but also other species (Afonso et al., 2019; Ahmed et al., 2019; Kalita et al., 2007). This shows the efficiency of the ISSR markers for the Brassicaceae family. Higher MI value (2.99) was obtained in comparison to Kalita et al. (2007). They also evaluated 28 Brassica accessions using 7 ISSR primers. However, Mahjoob et al. (2016) also revealed a higher MI value (6.4) than our study using 13 ISSR markers for 35 Brassica genera. The difference between MI values with respect to previous studies could be because of different accessions and primers used.

Rp is a parameter to determine the ability of a marker to separate the genotypes in the population. The Rp value higher than 1.50 points out a highly polymorphic character (Thakur et al., 2017a). The higher value of Rp is correlated with the number of fragments obtained from the marker. The reason for that is the formula of Rp is based on the cumulative I_b values. Our results for Rp ranges between 5.36 (UBC834 - UBC845) and 14.57 (UBC827). The mean value for overall markers was 8.29, which was really high with respect to previous studies (Gupta et al., 2014; Kalita et al., 2007; Singh et al., 2018; Teklewold & Becker, 2006; Thakur et al., 2017a), at which different species of Brassica genus were investigated.

The marker effectiveness was evaluated not only for all accessions but also specific to *B. juncea* and *B. arvensis* taxa. It was seen that ISSR markers used in the current study worked more effectively for *B. arvensis* (Rp = 7.1) than *B. juncea* (Rp = 4.2). This result is also supported by 4 NPBs for *B. arvensis* which can be used efficiently for the future analysis.

The population differentiation value (*Fst*) ranges between 0 and 1. A higher value means higher genetic differentiation between populations (Thakur et al., 2017a). Greater *Fst* value than 0.15 is an indicator of significant genetic differentiation (Frankham et al., 2010). In the present study, mean *Fst* value was computed as 0.4212 which was higher than previous studies (Chen et al., 2020; Ciancaleoni et al., 2018; Yousef et al., 2018) or similar to some of them (Sun et al., 2018; Tian et al., 2017). Likewise, the high value of expected heterozygosity is desirable for high genetic variation in the population. In previous studies, the mean value of expected heterozygosity was recorded as 0.088 (Takahashi et al., 2019) and 0.2883 (Wang et al., 2017). In the current study, the mean value of expected heterozygosity is higher than or similar to former studies which could be because of the substantial genetic diversity in the population.

ISSR technique is a combination of the benefits of AFLP and universality of RAPD. ISSRs are promising markers because of its longer primers (16-25 mers) with respect to RAPD primers (10-mers) that provides high annealing temperature (45-60℃) and reproducibility (Pradeep Reddy et al., 2002; Tarıkahya-Hacıoğlu, 2016). Mahjoob et al. (2016) compared different markers like ISSR, IRAP and REMAP for evaluation of genetic diversity in *Brassica sp.* and it was shown that the ISSR markers were the most effective one for genetic diversity among Brassicaceae family.

Overall, this study showed that an acceptable level of analysis for genetic diversity of Brassica genus can be applied via ISSR markers and this data can be used to improve new *Brassica* species through a breeding program.

Conclusion

Brassica genus is an important crop since it is used not only in industry but also as vegetables. To increase the gene pool and to target the specific desired traits for future breeding programs, using wild relatives is so crucial. For this reason, identifying the genetic relationship among taxa would be very enlightening to breeders. Here, genetic diversity of four major taxa of Brassica genus was investigated by using ISSR primers. In the present study, a high proportion of interspecific and intraspecific variation was observed. This can be useful for selecting the parental lines to create a roadmap to develop new mustard varieties.

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RESEARCH PAPER

Insight into effects of Axillarin and Verbascoside isolated from *Tanacetum alyssifolium* and *Plantago euphratica* on probiotic properties of *Lactobacillus acidophilus* and *Lactobacillus rhamnosus*

Nasip Ümran Turan [1], Hasan Ufuk Çelebioğlu [1,*], Hüseyin Akşit [2], Recep Taş [1]

[1]Bartın University, Faculty of Science, Department of Biotechnology, 74110 Bartın, Turkey.
[2]Erzincan Binali Yıldırım University, Faculty of Pharmacy, Department of Analytic Chemistry, 24100 Erzincan, Turkey

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***Corresponding Author**
Tel.: +90 505 241 7819
E-mail: ufukcelebioglu@gmail.com

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Abstract

Phenolic compounds are found in the seeds, flowers, leaves, stems, branches, and fruits of plants. They have an important place in human nutrition as many fruits and vegetables have phenolic compounds. Recently, many phenolic compounds have been used as functional foods and research of new phenolic compounds that are candidate to become functional foods is ongoing. Probiotics which are beneficial microorganisms, are living microbes that benefit the host when taken in sufficient amounts and they have an important role in human gastrointestinal microbiota. The present study aims to investigate effects of Axillarin and Verbascoside, two phenolic compounds isolated from *Tanacetum alyssifolium* and *Plantago euphratica,* respectively, on two very common probiotic bacteria *Lactobacillus acidophilus* and *Lactobacillus rhamnosus.* For this, growth kinetics, auto-aggregation, and microbial adhesion to solvents, which are some critical properties of probiotics, were investigated. Axillarin and verbascoside showed significant improvement on growths of *Lactobacillus acidophilus* LA-5 and *Lactobacillus rhamnosus* GG depending on the dose. Auto-aggregation properties were enhanced by these phenolic compounds, even though surface hydrophobicities were decreased. In conclusion, the present study indicates that the vital conditions of the probiotics could be modulated by Axillarin and/or Verbascoside, consequently their adhesion and colonization capabilities could be altered.

Introduction

Phenolic compounds are one of the highly studied topics for proving nature-based treatments. There are approximately 8000 phenolics present in plants, of which 4000 are elucidated (Bravo, 2009). Phenolic compounds are found in the seed flowers, leaves, stems, branches and fruits of plants. These compounds originated from pentose phosphate, shikimate, and phenylpropanoid pathways in plant metabolisms and are secondary metabolites in plants (Arceusz et al., 2013; Balasundram et al., 2006; Harborne et al., 1992; Silva et al., 2016). Phenolic compounds are effective in the growth, development, and the protection of plants against pathogenic organisms, giving taste and color properties to vegetables and fruits (Alasavar et al., 2001). These compounds, also called as polyphenols because of their structure, are found in many herbal foods and beverages, therefore they are present in significant amounts in human nutrition (Balasundram et al., 2006; lsavar et al., 2001). Polyphenols are thought to have many positive effects, including anti-allergic, anti-viral, antioxidant, anti-mutagenic, anti-carcinogenic, anti-inflammatory, anti-microbial, and anti-thrombotic activities (Balasundram et al., 2006; Moure et al., 2001). The health benefits of phenolic compounds are mostly related to their antioxidant and anti-inflammatory properties. Although phenolic compounds do not have a direct nutritional value, only 5-10% are absorbed in the small intestine and 90-95% pass into the large intestine

(Crozier et al., 2010). The compounds that interact directly with intestinal microflora may have positive or negative effects on these microorganisms (Cueva et al., 2017; Liano et al., 2016; Pereira-Caro et al., 2015). Probiotics are living microbes that benefit the host in sufficient amounts of practice and they have an important role in human nutrition and health. Such behavior of probiotics makes them functional foods that interact directly with gastrointestinal microflora (Arjmandi 2014; FAO, WHO 2006; Reuter 2001; Saarela et al., 2000). The most well-known probiotics belong to *Lactobacillus* genus. It is possible to find numerous studies regarding to modern-day *Lactobacillus acidophilus* and *Lactobacillus rhamnosus*. *Lactobacilli* are involved in human nutrition through their presence in milk and dairy products. Daily consumption of various types of food may alter the effects of probiotic bacteria on the intestinal system (Billoo et al., 2006; Kim et al., 2006; Lee & Salminen, 1995; Salminen et al., 1998).

The reported antibacterial and antioxidant properties of phenolic compounds and how these compounds affect probiotic microorganisms that benefit their hosts, are among the topics that are still being investigated and discussed among the community today. Verbascoside, which was isolated from the *Tanacetum alyssifolium* for this research, is phenylethanoid glycoside, belonging to the phenylpropanoid family. The other phenolic compound is Axillarin which is an o-methylated flavanol belonging to the Flavonoid group isolated from the *Plantago euphratica*. Previous studies on the phenolic compounds of Verbascoside and Axillarin (Figure 1) have been studied on their antioxidant and anti-inflammatory effects (Georgiev et al. 2012; Hu et al. 2017). Therefore, the present study aimed to elucidate the effects of these polyphenol compounds on probiotics microorganisms, *Lactobacillus acidophilus* and *Lactobacillus rhamnosus.*

Materials and Methods

Isolation of Axillarin

20 g of methanolic extract of *Tanacetum alyssifolium* was firstly fractioned over sephadex LH-20 using methanol as a mobile phase. According to the thin-layer chromatography (TLC) basis, Axillarin containing fractions (1.2 g) were combined and separated over C18 packed column. Water and methanol mixture were used as follows: 100:0, 80:20, 70:30 and 60:40 (v:v) each 0.5 L. Axillarin was obtained from fractions eluted with 60:40 water: methanol (v:v) system. The solvents were evaporated to dryness to give 875 mg of Axillarin.

Isolation of Verbascoside

Ten grams (10 g) of methanolic extract of *Plantago euphratica* was dissolved in hot water, then kept at +4°C overnight and non-soluble parts were removed by filtration. The water extract was lyophilized to give 4 g of pale-yellow solid. One gram (1 g) portion of extract was dissolved in 20 mL deionized water and repeatedly injected to HPLC ten times using 2 mL sample loop. The HPLC system used was a Shimadzu Prep-HPLC instrument with recycling mode equipped with LC20AR pump, SPD-20A UV-Vis detector and FRC-10A fraction collector. The mobile phase was a mixture of deionized water/acetonitrile (ACN); the UV wavelengths were 235 and 280 nm. The preparative column (250×20 mm, 10 µm) used was C18 (EMR Chrometsil). The flow rate was 8 mL/min. Verbascoside was purified from crude extract using a gradient elution from 90:10 to 50:50 (water: ACN) with 8 mL/min flow rate. The main peaks were collected according to the absorbance at 235 and 230 nm. Each collected fraction was purified using recycling mode with isocratic elution with 85:15 (Water: ACN), with 8 mL/min flow rate. The recycling process was continued until a clear separation was observed.

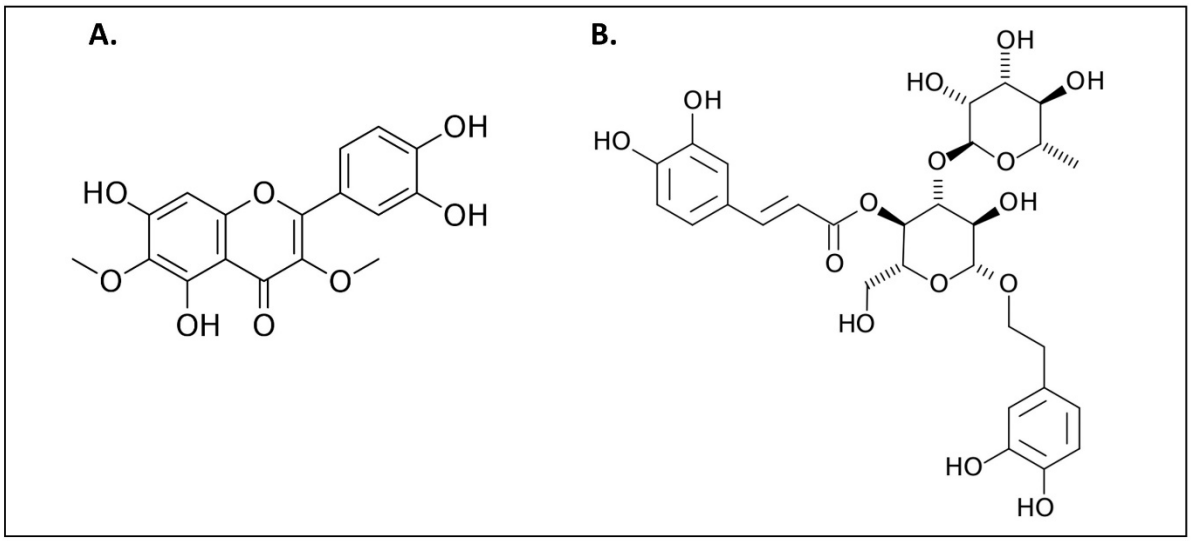

Figure 1. Chemical structures of (A) Axillarin and (B) Verbascoside.

^{1}H and ^{13}C NMR spectra were recorded on 400 MHz and 600 MHz, respectively (Bruker). Chemical shifts were in ppm from Me_4Si, generated from MeOD.

Growth of probiotic bacteria in the presence of Axillarin and Verbascoside, and their bacterial growth kinetics

Lactobacillus acidophilus LA-5 and *Lactobacillus rhamnosus* GG, kindly provided from Chr. Hansen, Turkey, were grown in Man, Rogosa and Sharpe (MRS) medium without shaking, at 37 °C (Celebioglu et al., 2018). The bacteria were treated separately with Axillarin and Verbascoside, not in mixture and Axillarin and Verbascoside were not added to the control groups (MRS only). Two different experiments were prepared for Axillarin and Verbascoside under the same conditions, and 5 µg/mL, 10 µg/mL and 12.5 µg/mL Axillarin and Verbascoside were added to bacterial growth media for each treatment groups. Bacterial optical density measurements were determined every four-hours with McFarland tube densitometer (Buch-Holm).

Probiotic auto-aggregation

Bacterial cells were incubated for 16 hours by treatment with Axillarin and Verbascoside, harvested in stationary phase (3200 g, 15 min), washed with Phosphate-saline buffer (PBS) and re-suspended in PBS to OD_{600} 0.5 (Kos et al., 2003). The percentage of auto-aggregation is calculated according to equation 1;

$$\textit{Auto-Aggregation\%} = \left(1 - \frac{At}{A0}\right) \times 100$$

where A_t is the absorbance measured after incubation and A_0 is the absorbance measured at 0^{th} hour (Kos et al., 2003).

Microbial adhesion to solvent (MATS)

Microbial adhesions of probiotic bacteria to solvents were measured by using the method described previously in the study of Kos et al. (2003). After growing, harvesting, and washing the bacterial cells as described above, they were suspended with 0.1 M KNO_3 (pH 6.2) to have OD_{600} of 0.5. One mL of Xylene (non-polar solvent) was added to 3 mL of bacterial suspension and incubated at room temperature (RT) for 10 min then the two-phase system was vortexed for 2 min, the aqueous phase was separated and incubated again for 20 min at RT. Absorbance was measured at 600 nm and bacterial adhesion solvent was calculated according to equation 2;

$$\textit{Adhesion \%} = \quad 1 - \left(\frac{A1}{A0}\right) x100$$

where, A_1 is the absorbance measured after the incubation and A_0 is the absorbance measured before the incubation (Kos et al., 2003).

Statistical analysis

Each experiment was designed as three biological replicates, and measurements were repeated three times. Results of measurements were expressed as mean ± standard deviation and One-way ANOVA was used to evaluate the results using GraphPad Prism version 8.0. Differences were considered as significant for $p<0.05$.

Results and Discussion

NMR assay of Axillarin

^{1}H NMR (600 MHz, MeOD) δ_H 7.61 (s, 1H), 7.52 (d, *J* = 8.0 Hz, 1H), 6.89 (d, *J* = 8.0 Hz, 1H), 6.48 (s, 1H), 4.84 (brs, 4H, -OH), 3.87 (s, 3H, -OCH_3), 3.78 (s, 3H, -OCH_3). ^{13}C NMR (150 MHz, MeOD) δ_C 178.8 (C-4),157.4 (C-7), 156.7 (C-2), 152.3 (C-5), 152.2 (C-9), 148.5 (C-4'), 145.0 (C-3'), 137.8 (C-3), 131.2 (C-6), 121.5 (C-1'), 120.9 (C-6'), 115.1 (C-5'), 115.0 (C-2'), 104.9 (C-10), 93.6 (C-8),59.5 (C-6-OCH_3), 59.1 (C-3-OCH_3).

NMR assay of Verbascoside

^{1}H NMR (400 MHz, MeOD) δ_H 7.62 (d, J=15.87, 1H, Hβ'), 7.08 (d, J=2.12, 1H, H2'), 6.98 (dd, J=8.21, 2.12, 1H, H6'), 6.80 (d, J=8.09, 1H, H5'), 6.72 (d, J=2.09, 1H, H2), 6.70 (d, J=7.91, 1H, H5), 6.59 (dd, J=8.00, 2.14, 1H, H6), 6.30 (d, J=15.88, 1H, Hα'), 5.21 (d, J=1.83, 1H, H1'''), 4.94 (m, 1H, H4''), 4.40 (d, J=7.86, 1H, H1''), 4.07 (dt, J=9.71, 7.32, 1H, Hαa), 3.94 (m, 1H, H2'''), 3.84 (t, J=9.20, 1H, H3''), 3.74 (m, 1H, Hαb), 3.65 (m, 1H, H6''a), 3.61 (m, 1H, H3'''), 3.58 (m, 1H, H5'''), 3.56 (m, 1H, H5''), 3.54 (m, 1H, H6''b), 3.41 (t, J=8.48, 1H, H2''), 3.32 (m, 1H, H4'''), 2.81 (dt, J=7.35, 2.45, 2H, Hβ), 1.11 (d, J=6.21, 3H, H6'''). ^{13}C NMR (101 MHz, MeOD) δ_C 166.93 (C=O), 148.38 (C4'), 146.63 (Cβ'), 145.43 (C3'), 144.74 (C3), 143.28 (C4), 130.15 (C1), 126.32 (C1'), 121.83 (C6'), 119.91 (C6), 115.76 (C2), 115.17 (C5'), 114.96 (C5), 113.91 (C2'), 113.37 (Cα'), 102.83 (C1''), 101.63 (C1'''), 80.27 (C3''), 74.83 (C2''), 74.65 (C5''),

NMR assignments were fully agreed with literature for Verbascoside and Axillarin (Akdemir et al., 2004). Verbascoside is a common phenyl ethanoid for *Plantago* species and was previously isolated from *P. lagopus* (Harput et al., 2012), *P. pellardii* (Gálvez et al., 2005), and *P. cynops* (Debrauwer et al., 1989).

Bacterial growth kinetics

In this study, Axillarin and Verbascoside were used in concentrations of 5, 10 and 12.5 µg/mL. The groups treated with Axillarin and Verbascoside showed significant improvement on *Lactobacillus acidophilus* LA-5 and *Lactobacillus rhamnosus* GG depending on the dose of phenolic compounds, when compared with control groups (Figure 2). Previous studies have examined the antimicrobial effect of Axillarin and found that it inhibited the development of pathogenic bacteria (Avila et al., 1999). However, in the present study, Verbascoside have positively influenced growth of *Lactobacillus acidophilus* and *Lactobacillus rhamnosus*.

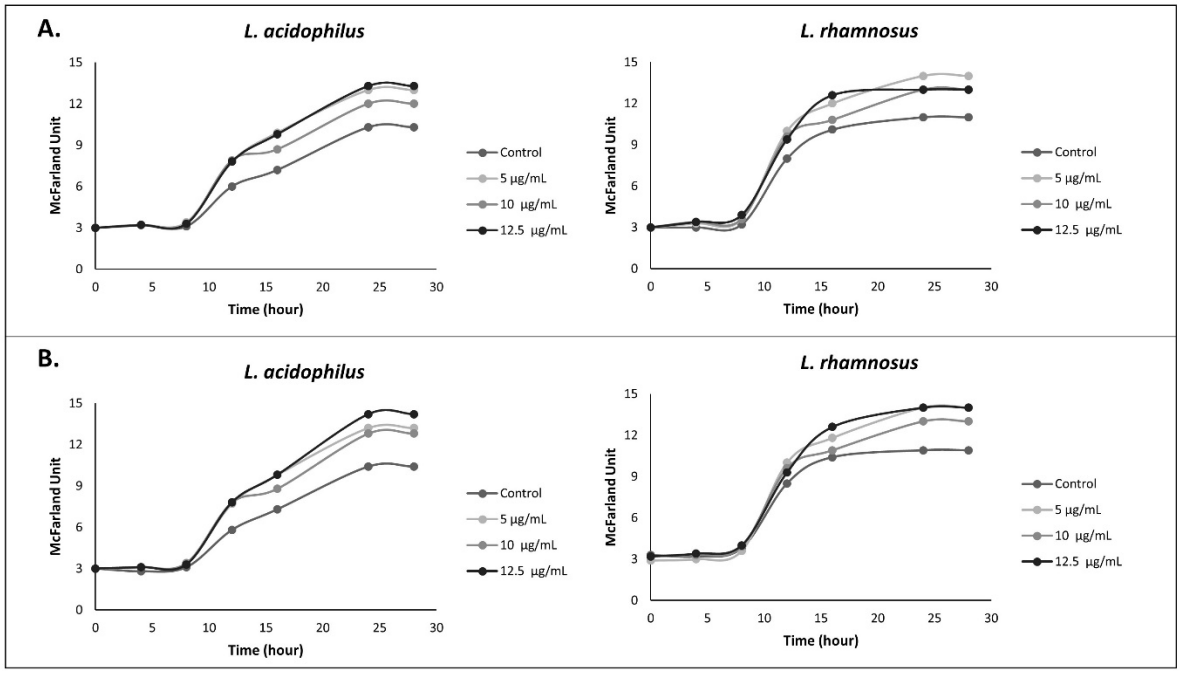

Figure 2. Growth kinetics of probiotic bacteria. (A) Growth curve of *Lactobacillus acidophilus* LA-5 and *Lactobacillus rhamnosus* GG when grown in the presence of Axillarin. (B) Growth curve of *Lactobacillus acidophilus* LA-5 and *Lactobacillus rhamnosus* GG when grown in the presence of Verbascoside.

Anti-inflammatory effect studies have also been reported for Axillarin, but on *Lactobacillus acidophilus* LA-5 and *Lactobacillus rhamnosus* GG have shown an effect that promoted development (Beg et al., 2011). The fact that these compounds have no antimicrobial effects on *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* suggests that they have the potential to have selectively positive effects on beneficial bacteria in the intestinal microflora.

Bacterial auto-aggregation

Even though there are many protective mechanisms present in the gastrointestinal mucosa, humans are occasionally exposed to enteric pathogens. Enteric pathogens cause infection by colonizing on microflora present in the gastrointestinal tract (Sekirov et al., 2010). Therefore, adhesion to intestinal epithelial cells is a prerequisite for colonizing probiotic strains in the gastrointestinal tract, preventing their immediate elimination (Alander et al., 1997; Freter 1992; Pedersen and Tannock 1989). Consequently, auto-aggregation is an important condition for the adhesion and survival of probiotic strains to the intestinal epithelium (Boris et al., 1997; Del Re et al., 1998). The concentration of 5 µg/mL of Axillarin significantly decreased the auto-aggregation of *Lactobacillus acidophilus* at 1st hour, then the next hours of the auto-aggregation, it significantly (p<0.05) increased the auto-aggregation, when compared to

Table 1. Auto-aggregation percentages of probiotics grown in the presence of Axillarin. The results are given as mean with standard deviations. Asterisks (*) indicate the differences are statistically significant (p<0.05) when compared to respective control groups, according to One-Way ANOVA.

	1st Hour	2nd Hour	3rd Hour	4th Hour	5th Hour
			L. acidophilus		
Control	59.2 ± 0.6	21.8 ± 0.7	59.7 ± 13.0	70.4 ± 17.6	83.8 ± 9.5
5 µg/mL	20.5 ± 1.4 *	42.7 ± 1.3 *	70.4 ± 4.5 *	79.1 ± 1.5 *	81.6 ± 0.7
10 µg/mL	21.8 ± 0.3 *	65.0 ± 0.2 *	70.1 ± 0.3 *	72.1 ± 0.5	84.1 ± 7.0
12.5 µg/mL	29.6 ± 0.2 *	32.9 ± 0.1 *	64.2 ± 3.8	79.8 ± 2.0 *	87.4 ± 6.1 *
			L. rhamnosus		
Control	84.5 ± 2.6	N.D.	N.D.	N.D.	N.D.
5 µg/mL	84.3 ± 2.4	N.D.	N.D.	N.D.	N.D.
10 µg/mL	88.7 ± 5.2 *	N.D.	N.D.	N.D.	N.D.
12.5 µg/mL	89.1 ± 3.0 *	N.D.	N.D.	N.D.	N.D.

N.D.: Not Determined

control (Table 1). The second concentration is 10 µg/mL and showed a significant decrease at 1st hour, but at 2nd and 3rd hour, it increased the auto-aggregation, compared to control group. The last concentration is 12.5 µg/mL and showed a significant decrease in the first two hours but increase in auto-aggregation in the last hours. This could mean Axillarin has a positive effect on auto-aggregation of *Lactobacillus acidophilus*. On the other hand, the auto-aggregation of *Lactobacillus rhamnosus* was significantly increased by 10 µg/mL and 12.5 µg/mL of Axillarin in the first hour as compared to control group. In the next hours of the assay, the auto-aggregation was reached to the maximum, which means the comparison and measurement were not possible (stated in the table as ND). The results of auto-aggregation indicate that these polyphenols can positively affect the adhesion of the probiotic bacteria as auto-aggregation which is very important for probiotic microorganisms to adhesion and live in the gastrointestinal tract (GIT) (Boris et al., 1997; Del Re et al., 1998).

Auto-aggregation of Verbascoside showed a significant decrease (p<0.05) in auto-aggregation of *L. acidophilus* at the concentration of 5 µg/mL in the 1st and 2nd hours as compared with control groups; however, increase in the 5th hour (Table 2). The concentration of 10 µg/mL showed an important decrease in auto-aggregation in the 1st and 2nd, but significantly increased in the 3rd and 5th hours. The concentration of 12.5 µg/mL significantly decreased auto-aggregation in the 2nd and there are significantly increases at 3rd, 4th, and 5th hours when compared with control group. On the other hand, auto-aggregation of *Lactobacillus rhamnosus* was significantly increased by Verbascoside with every concentration. In the next hours of measurements, the auto-aggregations were not able to be obtained, which means there were no bacteria found on the surface of suspension because they were aggregating completely down to the tubes (Table 2).

Auto-aggregation of probiotics grown in the presence of phenolic compounds was mostly lower than the control group at 1st hour. This could be explained by that bacteria started interacting each other at the beginning and after one hour, these interactions could not be fully achieved. Thus, in time, bacteria could interact fully, and the effects of phenolic compounds could be observed at the later hours. Increased auto-aggregation, at later hours, can indicate these beneficial bacteria are positively affected by Axillarin and Verbascoside, thus they have potential to adhere more in the GIT and show their probiotic activities to the host (Boris et al., 1997; Del Re et al., 1998).

Microbial adhesion to solvents (MATS)

The surface properties of *Lactobacilli* contribute to their interactions with the host and gastrointestinal microbiota. Thus, this can affect their residence in GIT by allowing *Lactobacilli* adhering to intestinal tissue, as well as affecting interactions with their own species and other bacteria (Kleerebezem et al., 2010; Lebeer et al., 2008). The adhesion behavior of microbial cells has been shown to depend on the van der Waals interactions and balance of electrostatic on the hydrophobic character of the surfaces (Boonaert & Rouxhet 2000). Hydrophobicity plays an important role for the contact between a bacterial cell and mucous or epithelial cell (Schillinger et al., 2005). In this assay, Xylene (non-polar solvent) was used to assess the hydrophobic and hydrophilic characteristics of bacterial surface (Bellon-Fontaine et al., 1996; Kos et al., 2003). As a result of the assays conducted, surface hydrophobicity of both bacteria was significantly (p<0.05) decreased by presence of Axillarin and Verbascoside at every concentration, as compared to control (Figure 3).

Table 2. Auto-aggregation percentages of probiotics grown in the presence of Verbascoside. The results are given as mean with standard deviations. Asterisks (*) indicate the differences are statistically significant (p<0.05) when compared to respective control groups, according to One-Way ANOVA applied for each column.

	1st Hour	2nd Hour	3rd Hour	4th Hour	5th Hour
			L. acidophilus		
Control	31.4 ± 12.2	47.5 ± 13.2	47.4 ± 1.1	69.7 ± 7.9	64.5 ± 3.6
5 µg/mL	23.1 ± 7.9 *	17.1 ± 4.8 *	49.3 ± 1.9	63.2 ± 5.3	71. 5 ± 0.5 *
10 µg/mL	28.0 ± 2.3 *	11.6 ± 1.3 *	61.8 ± 0.2 *	68.2 ± 0.7	77.0 ± 10.1 *
12.5 µg/mL	30.4 ± 0.9	34.6 ± 6.9 *	65.0 ± 14.6 *	72.9 ± 0.1 *	62. 7 ± 0.6
			L. rhamnosus		
Control	65.8 ± 2.1	N.D.	N.D.	N.D.	N.D.
5 µg/mL	80.5 ± 13.4 *	N.D.	N.D.	N.D.	N.D.
10 µg/mL	84.6 ± 13.0 *	N.D.	N.D.	N.D.	N.D.
12.5 µg/mL	81.0 ± 0.6 *	N.D.	N.D.	N.D.	N.D.

N.D.: Not Determined

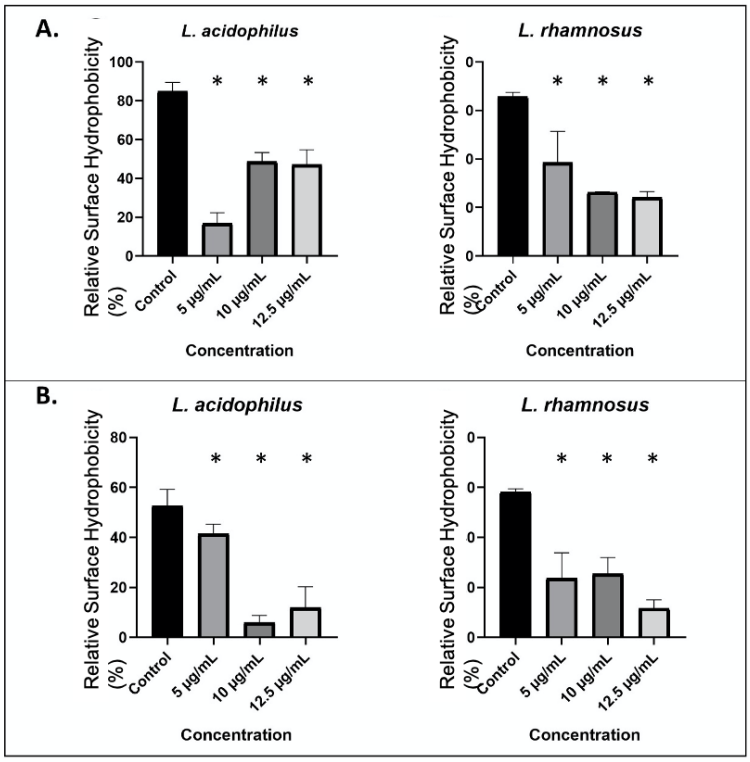

Figure 3. Surface hydrophobicity (A) of *Lactobacillus acidophilus* LA-5 and *Lactobacillus rhamnosus* GG when grown in the presence of Axillarin and (B) of *Lactobacillus acidophilus* LA-5 and *Lactobacillus rhamnosus* GG when grown in the presence of Verbascoside. Asterisks (*) indicate the differences are statistically significant (p<0.05) when compared to respective control groups, according to One-Way ANOVA.

Lactobacillus may well serve as model systems for the study of structure-property functions of the bacterial cell envelope (Schär-Zammaretti & Ubbink 2003). Physicochemical properties of the cell surface, such as hydrophobicity, can affect the automatic aggregation and adhesion of bacteria to different surfaces. Cell adhesion is a complex process involving contact between bacterial cell membrane and interacting surfaces (Del Re et al., 1998; Perez et al., 1998; Wadström et al., 1987). On this topic, reported investigations were on the structure and forces of interaction and composition related to bacterial adhesion to intestinal epithelial cells (Del Re et al., 1998; Perez et al., 1998) and mucus (Collado et al., 2005). However, in addition to surface properties of the bacteria, the surface proteins that many *lactobacilli* possess, specifically S-layer proteins binding to the cell wall in a non-covalent manner, play significant roles in adhesion (Celebioglu & Svensson, 2017). The biological functions of the S-layer include protection, determination of cell shape, molecular and ion capture to surfaces where adhesion takes place. It is thought that the structure responsible for the attachment of a bacterial cell to the intestinal epithelium is the S-layer (Meng et al., 2017; Wasko et al., 2014). Therefore, not only hydrophobicity and aggregation are sufficient for good adhesion ability, but more importantly, surface proteins of bacteria play a key role on bacterial adhesion (Schär-Zammaretti & Ubbink 2003; Sengupta et al., 2013).

Conclusion

In the present study, growth kinetics, auto-aggregation, and microbial adhesion to solvents assays were tested using Axillarin and Verbascoside polyphenols on probiotics, which are beneficial microorganisms to the host (*Lactobacillus acidophilus* LA-5 and *Lactobacillus rhamnosus* GG). Probiotic bacteria tested here, treated with Axillarin and Verbascoside polyphenols, showed significant alterations in auto-aggregation and hydrophobicity experiments. The results from the auto-aggregation and hydrophobicity (MATS) experiments indicate that the vital conditions of the probiotics could be modulated by Axillarin and/or Verbascoside, consequently alter their adhesion and colonization capabilities. Furthermore, future studies could investigate how polyphenols affect the surface proteins of probiotic bacteria, which are of great importance for bacterial adhesion.

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