Food Science and Biotechnology (2025) 34:2249–2253
https://doi.org/10.1007/s10068-025-01850-x

RESEARCH NOTE

Development of a nitrate-responsive whole-cell biosensor based on engineered probiotic *Saccharomyces boulardii*

Woohyuk Lee[1,2] · Sang-Hun Do[1,2] · Jonghyeok Shin[3] · Sun-Ki Kim[1,2]

Received: 3 January 2025 / Revised: 21 February 2025 / Accepted: 27 February 2025 / Published online: 12 March 2025

Abstract

This study aimed to construct a probiotic *Saccharomyces boulardii*-based biosensor that can sense and respond to nitrate, a biomarker of gut inflammation. To this end, a portion of the nitrate assimilation pathway in *Ogataea polymorpha* required for the designed nitrate-responsive genetic circuit was introduced into *S. boulardii*, which lacks the corresponding pathway. Specifically, the genes involved in nitrate transport (*YNT1*) and nitrate-responsive transcriptional activation (*YNA1* and *YNA2*) were introduced into *S. boulardii* using the CRISPR/Cas9-based genome editing system to construct the SKSC499 strain. Next, we investigated whether *YNT1*, *YNI1*, and *YNR1* promoters, which are activated by nitrate-bound YNA1/YNA2 transcription factor in *O. polymorpha*, induced expression of the reporter gene (G418 resistance marker) in the SKSC499 strain in a nitrate concentration-dependent manner. Among the candidate promoters, the *YNR1* promoter effectively induced growth of the SKSC499 strain only when nitrate was present in the medium containing the G418 antibiotic.

Keywords *Saccharomyces boulardii* · Whole-cell biosensor · Gut inflammation · Genetic circuit

Introduction

The prevalence of inflammatory bowel disease (IBD), defined as a chronic inflammatory and ulcerative process affecting the intestines, has increased significantly in recent years (Kuenzig et al., 2022). Because IBD is associated with the pathogenesis of colorectal cancer, it is important to diagnose IBD at an early stage (Fantini and Guadagni, 2021). IBD biomarkers include reactive oxygen species (ROSs), reactive nitrogen species (RNSs), thiosulfate ($S_2O_3^{2-}$), and tetrathionate ($S_4O_6^{2-}$) (Kamdar et al., 2016; Simmonds and Rampton, 1993). In particular, gut inflammation induces high-level expression of nitric oxide synthase (NOS), leading to an increase in the production of nitric oxide (NO) (Stanek et al., 2008). Nitrate (NO_3^-) is an important biomarker of IBD because NO (i.e., a transient product of RNS) is readily converted to nitrate (Saijo et al., 2010; Szabó et al., 2007). Accordingly, a nitrate-responsive genetic circuit consisting of the NarX-NarL two-component regulatory system was constructed previously in a probiotic *Escherichia coli* for living diagnostics for gut inflammation, whose performance was validated in mice with colitis (Woo et al., 2020).

Recent advances in the design-build-test-learn cycle of synthetic biology have accelerated the application of engineered bacteria reprogrammed using advanced genetic circuits for living diagnostics and therapeutics (Gurdo et al., 2023). Probiotics, in particular, act as a chassis reprogrammed with genetic circuits to detect disease biomarkers and efficiently deliver therapeutics in a site-specific manner (Vo et al., 2019). While most research has focused on probiotic bacteria, the probiotic yeast *Saccharomyces*

Woohyuk Lee and Sang-Hun Do contributed equally to this work.

✉ Sun-Ki Kim
skkim18@cau.ac.kr

Woohyuk Lee
mercury0610@cau.ac.kr

Sang-Hun Do
roo00700@naver.com

Jonghyeok Shin
shin9114@kribb.re.kr

[1] Department of Food Science and Biotechnology, Chung-Ang University, Anseong, Gyeonggi 17546, Republic of Korea

[2] GreenTech-Based Food Safety Research Group, BK21 Four, Chung-Ang University, Anseong, Gyeonggi 17546, Republic of Korea

[3] Synthetic Biology Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon 34141, Republic of Korea

cerevisiae var. *boulardii* has recently received substantial attention for its ability to effectively relieve diarrhea and gut inflammation (Kelesidis and Pothoulakis, 2012). However, an engineered *S. boulardii* capable of sensing nitrate has not yet been developed because the nitrate assimilation pathway does not exist in *S. boulardii* (Khatri et al., 2017; Perli et al., 2021).

As a few yeast species, including *Ogataea* (*Hansenula*) *polymorpha*, can assimilate nitrates (Fig. 1A) (Siverio, 2002), the genes involved in nitrate transport (*YNT1*) and nitrate-responsive transcriptional activation (*YNA1* and *YNA2*) were introduced into *S. boulardii*. In addition, the expression of the reporter gene (G418 resistance marker, $G418^R$) is designed to be under the transcriptional control of the nitrate-responsive promoter (Fig. 1B). As a result, the engineered *S. boulardii* strain showed growth only in the presence of nitrate in antibiotic-supplemented media, demonstrating that it can act as a whole-cell biosensor to detect nitrate.

Materials and methods

Strains and plasmids

E. coli TOP10 (Invitrogen, Carlsbad, CA, USA) was used for gene cloning. *S. boulardii* ATCC MYA-796 was used as the chassis for the whole-cell biosensor. The genomic DNA of *O. polymorpha* ATCC MYA-336 was used for the amplification of genes involved in nitrate assimilation. The strains and plasmids used in this study are listed in Table S1.

Genetic manipulation

The primer sets used for amplification of the genes involved in nitrate assimilation, yeast episomal plasmids, and guide RNA (gRNA) plasmids are listed in Table S2. The resulting PCR products were combined using NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs, Ipswich, MA, USA), as specified by the manufacturer.

Episomal plasmids containing the genes coding for the geneticin (G418) resistance marker ($G418^R$) were transformed into *S. boulardii* ATCC MYA-796 using a yeast EZ-transformation kit (MP Biomedicals, Santa Ana, CA, USA). Transformants were isolated on solid YP20D medium (10 g/L yeast extract, 20 g/L Bacto™ Peptone, 20 g/L

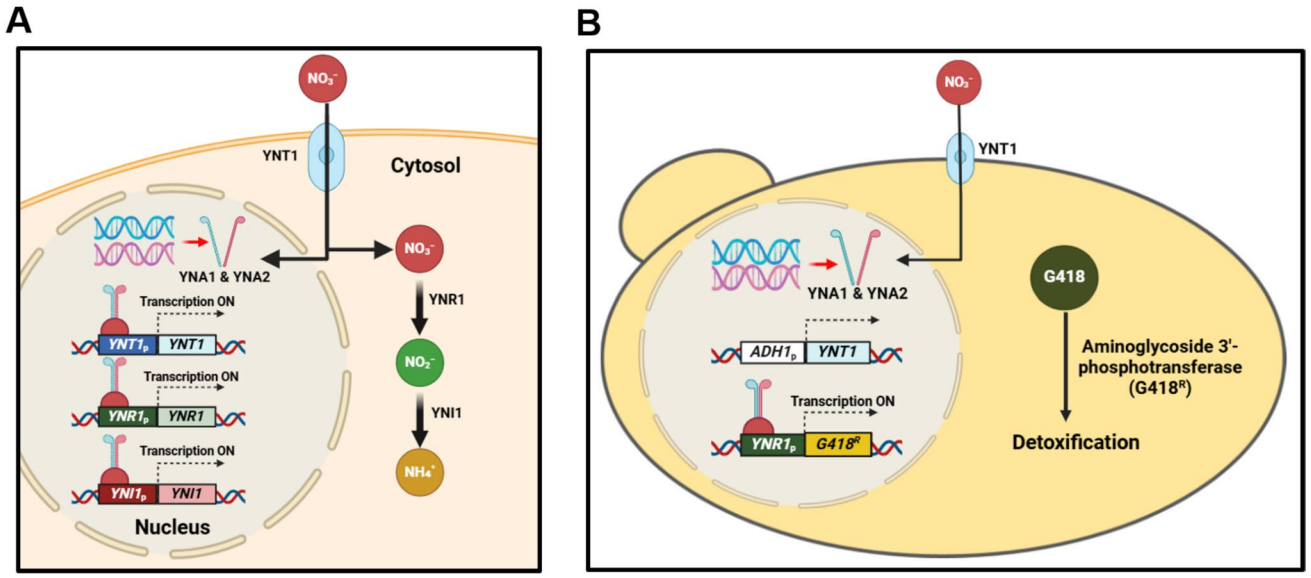

Fig. 1 Schematic illustration representing the nitrate assimilation pathway present in *Ogataea polymorpha* (**A**) and the designed nitrate-responsive genetic circuit in *Saccharomyces boulardii* (**B**). (**A**) In *O. polymorpha*, nitrate (NO_3^-) is transported into the cell by nitrate transporter (YNT1) and then binds to nitrate-responsive transcriptional activators (YNA1/YNA2) in the nucleus. After binding to nitrate, the YNA1/YNA2 heterodimer activates *YNT1*, *YNI1*, and *YNR1* promoters and induces gene expression. Nitrate is converted to nitrite (NO_2^-) by the reaction catalyzed by nitrate reductase (YNR1), and then nitrite is converted to ammonia (NH_4^+) by the reaction catalyzed by nitrite reductase (YNI1). (**B**)*YNA1*, *YNA2*, and *YNT1* genes from *O. polymorpha* were constitutively expressed in the *S. boulardii* SKSC499 strain under the transcriptional control of the *GPD*, *PGK1*, and *ADH1* promoters, respectively. In addition, the expression of the gene coding for the G418 resistance marker ($G418^R$) was designed to be under the transcriptional control of the *YNR1* promoter

glucose, and 15 g/L Bacto™ Agar) containing 300 μg/mL aureobasidin A.

The *YNA1* (*GPDp-YNA1-CYC1t*), *YNA2* (*PGKp-YNA2-CYC1t*), and *YNT1* (*ADH1p-YNT1-CYC1t*) expression cassettes were introduced into the CS8 (Kwak et al., 2017), CS12, and CS6 (Park et al., 2023) loci, respectively, using the genome editing system based on clustered regularly interspaced palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9), as previously described (Liu et al., 2016). In *S. boulardii*, the CS8, CS12, and CS6 are intergenic regions located, respectively, between *YPR015C* and *YPR014C* on chromosome XVI, *LYS14* and *YDR034C-D* on chromosome IV, and *HIP1* and the gene coding for tRNA on chromosome VII.

Media and culture conditions

E. coli was grown at 37 °C and 250 rpm for 12 h in Luria–Bertani medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) containing 50 μg/mL ampicillin for gene cloning. Engineered *S. boulardii* strains were pre-cultured at 30 °C and 250 rpm for 48 h in YP20D medium (10 g/L yeast extract, 20 g/L Bacto™ Peptone, and 20 g/L glucose) containing 300 μg/mL aureobasidin A. The pre-cultured cells were harvested and inoculated into main cultures with an initial optical density at 600 nm (OD_{600}) of 1.0. The main fermentation experiments to confirm that $G418^R$ is not expressed in the absence of nitrate were performed at 30 °C and 250 rpm for 120 h in a test tube containing 5 mL of YP20D medium with various concentrations (0, 200, and 400 μg/mL) of G418. The main fermentation experiments to confirm the nitrate-responsive expression of $G418^R$ were performed at 30 °C and 250 rpm for 120 h in a test tube containing 5 mL of YP20D medium with 200 μg/mL G418 and various concentrations (0, 50, and 100 mM) of sodium nitrate ($NaNO_3$).

Results and discussion

Construction of the nitrate-responsive genetic circuit in *S. boulardii*

In *O. polymorpha*, the genes responsible for nitrate assimilation are organized in a cluster, which includes a high-affinity nitrate transporter (*YNT1*), nitrate reductase (*YNR1*), nitrite reductase (*YNI1*), and transcriptional activators (*YNA1* and *YNA2*) (Siverio, 2002). YNA1 and YNA2 form a heterodimer and can only bind to the upstream activating sequence (UAS) in the presence of nitrate to drive expression of the *YNT1*, *YNR1*, and *YNI1* genes (Fig. 1A) (Ávila et al., 1998). Although the sulfite efflux permease (SSU1) is known to export nitrite and nitrate extracellularly in *S. cerevisiae*, a transporter that can transport nitrate into the cell is yet to be reported (Cabrera et al., 2014). Therefore, the expression cassettes containing the *YNT1, YNA1*, and *YNA2* genes under the transcriptional control of constitutive promoters were introduced into the *S. boulardii* chromosome (designated here as SKSC499) using the CRISPR/Cas9 system (Fig. 1B). To ensure that the gene circuit drives expression of the reporter gene ($G418^R$) only in the presence of nitrate, the $G418^R$ gene was designed to be under the transcriptional control of YNT1p, YNI1p, and YNR1p (the *YNT1*, *YNI*1, and *YNR1* promoters, respectively). We note that YNT1p, YNI1p, and YNR1p contain the nitrate-UAS that can recruit the YNA1/YNA2 transcriptional activator in the presence of nitrate to drive expression of the downstream reporter gene (Silvestrini et al., 2015). Although YNT1p, YNI1p, and YNR1p drive gene expression in response to nitrate concentration in *O. polymorpha*, predicting which promoter will work properly in the heterologous strain *S. boulardii* is difficult. Therefore, we designed the expression of the $G418^R$ gene to be transcriptionally regulated by one of the three promoters (Fig. 2).

Validation of the designed genetic circuit in the engineered *S. boulardii*

For the heterologous YNT1p, YNI1p, and YNR1p to work properly in *S. boulardii*, they must first not induce expression of their downstream genes in the absence of nitrate. Accordingly, we investigated whether the expression of the $G418^R$ gene is induced in the absence of nitrate. As shown in Fig. 2, the three promoters did not drive the $G418^R$ expression in the absence of nitrate; hence, none of the *S. boulardii* strains were able to grow in the medium containing the antibiotic G418. Next, the plasmids containing the *YNT1p-*$G418^R$, *YNI1p-*$G418^R$, and *YNR1p-*$G418^R$ expression cassettes were introduced into the SKSC499 strain containing *YNT1*, *YNA1*, and *YNA2* genes in its genome (Table S1). The SKSC499 strain harboring the *YNT1p-*$G418^R$ or *YNI1p-*$G418^R$ expression cassette was able to grow in the medium containing 200 μg/mL G418 even in the absence of nitrate (Fig. 3). Considering that the wild-type *S. boulardii* strain harboring the *YNT1p-*$G418^R$ or *YNI1p-*$G418^R$ expression cassette could not grow under the same condition (Fig. 2), this finding suggested that the heterologous YNA1/YNA2 transcriptional activator was functionally expressed in *S. boulardii*, leading to activation of YNT1p and YNI1p. However, the two promoters cannot be used as a component of the nitrate-responsive genetic circuit because they drive the expression of $G418^R$ regardless of the presence of nitrate. Notably, the YNR1p induced expression of $G418^R$ in the SKSC499 strain containing the other components (i.e., the *YNT1, YNA1*, and *YNA2* genes) of the nitrate-responsive genetic circuit. Specifically, the SKSC499 strain harboring the *YNR1p-*$G418^R$

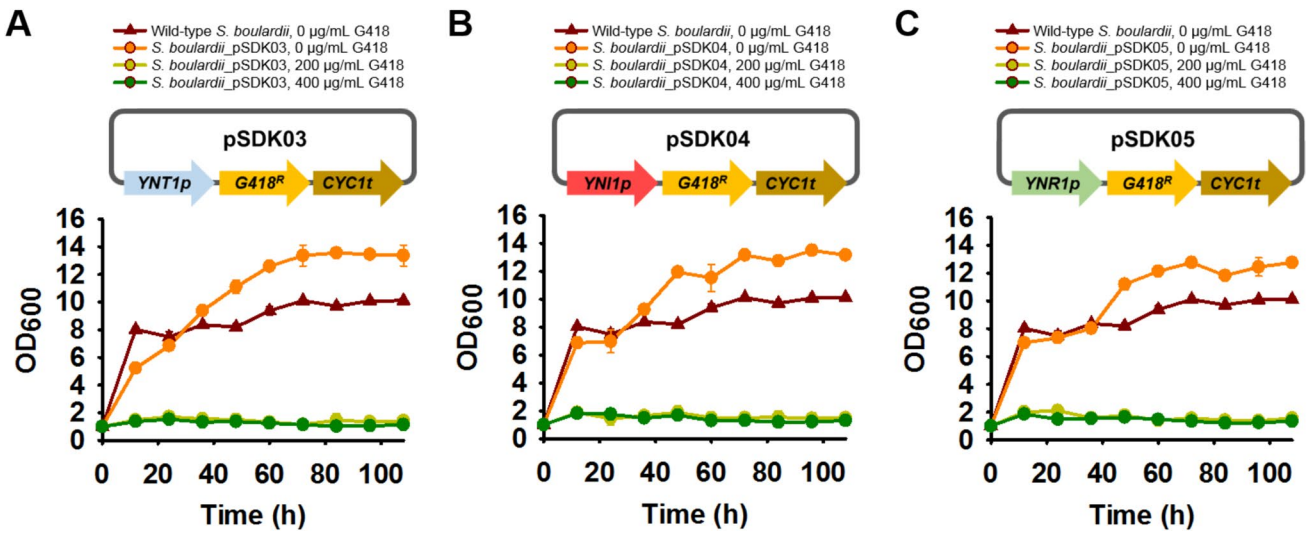

Fig. 2 Growth curves of the wild-type *Saccharomyces boulardii* and *S. boulardii* strains harboring pSDK03 (**A**), pSDK04 (**B**), and pSDK05 (**C**) in the medium containing various concentrations of G418. Results represent the mean of two experiments, with error bars indicating standard deviation

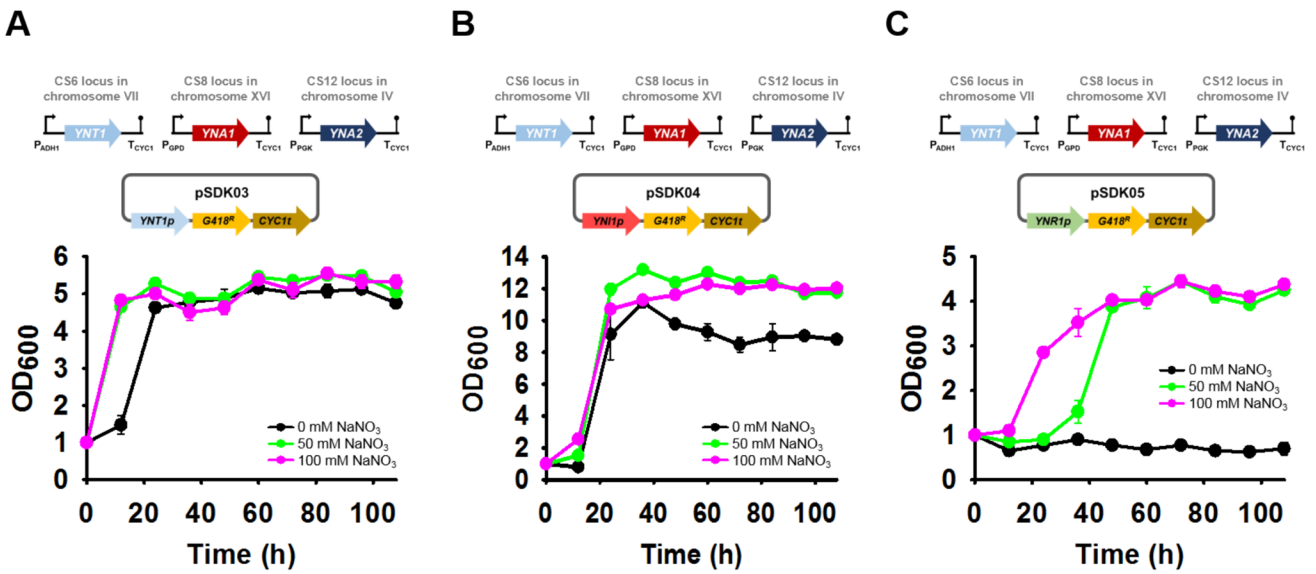

Fig. 3 Growth curves of the SKSC499 strain harboring pSDK03 (**A**), pSDK04 (**B**), and pSDK05 (**C**) in the medium containing 200 μg/mL G418 and various concentrations of sodium nitrate ($NaNO_3$). Results represent the mean of two experiments, with error bars indicating standard deviation

expression cassette exhibited growth only in the presence of nitrate (Fig. 3). Although the nitrate-responsive genetic circuit was successfully constructed in *S. boulardii* by introducing the *YNT1, YNA1*, and *YNA2* genes along with the *YNR1p-G418*^{R} expression cassette, relatively high concentrations of nitrate were required to detect the presence of nitrate using growth as a signal. Because the expression of antibiotic resistance markers must be sufficient for the cells to grow in the presence of antibiotics, higher levels of expression of the reporter gene will likely be required than if an alternative type of signal, such as fluorescence or bioluminescence, was used. This limitation could be overcome by replacing the *G418*^{R} gene with a firefly luciferase reporter gene that generates a bioluminescent signal (Park et al., 2023). In this study, the *G418*^{R} gene was used as the reporter whose expression is induced by nitrate, with the potential for future substitution with anti-inflammatory factors, such as tumor necrosis factor-alpha (TNF-α) (Ruder et al., 2019) and interleukin-10 (IL-10) (Li and He, 2004), to selectively express the anti-inflammatory factor only when

inflammation develops in the colon, thereby alleviating inflammation.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10068-025-01850-x.

Acknowledgements This study was financially supported by the National Research Foundation of Korea Grant (2022R1C1C1003154 and RS-2024-00440975), funded by the Korean Ministry of Science, ICT, and Future Planning. Additional support was provided by the Chung-Ang University Graduate Research Scholarship in 2024.

Funding Korean Ministry of Science, ICT, and Future Planning, 2022R1C1C1003154, Sun-Ki Kim, RS-2024-00440975, Sun-Ki Kim, Chung-Ang University Graduate Research Scholarship.

Declarations

Competing interests Sun-Ki Kim, who is the Managing Editor of *Food Science and Biotechnology*, was not involved in the editorial consideration of this manuscript. Other than that, the authors declare no conflict of interest.

References

Ávila J, González C, Brito N, Siverio JM. Clustering of the *YNA1* gene encoding a $Zn(II)_2Cys_6$ transcriptional factor in the yeast *Hansenula polymorpha* with the nitrate assimilation genes *YNT1*, *YNI1* and *YNR1*, and its involvement in their transcriptional activation. Biochemical Journal. 335: 647-652 (1998)

Cabrera E, Gonzalez-Montelongo R, Giraldez T, Alvarez de la Rosa D, Siverio JM. Molecular components of nitrate and nitrite efflux in yeast. Eukaryotic Cell. 13: 267-278 (2014)

Fantini MC, Guadagni I. From inflammation to colitis-associated colorectal cancer in inflammatory bowel disease: Pathogenesis and impact of current therapies. Digestive and Liver Disease. 53: 558-565 (2021)

Gurdo N, Volke DC, McCloskey D, Nikel PI. Automating the design-build-test-learn cycle towards next-generation bacterial cell factories. New Biotechnology. 74: 1-15 (2023)

Kamdar K, Khakpour S, Chen J, Leone V, Brulc J, Mangatu T, Antonopoulos DA, Chang EB, Kahn SA, Kirschner BS, Young G, DePaolo RW. Genetic and metabolic signals during acute enteric bacterial infection alter the microbiota and drive progression to chronic inflammatory disease. Cell Host & Microbe. 19: 21-31 (2016)

Kelesidis T, Pothoulakis C. Efficacy and safety of the probiotic *Saccharomyces boulardii* for the prevention and therapy of gastrointestinal disorders. Therapeutic Advances in Gastroenterology. 5: 111-125 (2012)

Khatri I, Tomar R, Ganesan K, Prasad GS, Subramanian S. Complete genome sequence and comparative genomics of the probiotic yeast *Saccharomyces boulardii*. Scientific Reports. 7: 371 (2017)

Kuenzig ME, Fung SG, Marderfeld L, Mak JWY, Kaplan GG, Ng SC, Wilson DC, Cameron F, Henderson P, Kotze PG, Bhatti J, Fang V, Gerber S, Guay E, Jayawarden SK, Kadota L, Maldonado DF, Osei JA, Sandarage R, Stanton A, Wan M, InsightScope Pediatric IBD Epidemiology Group, Benchimol EI. Twenty-first century trends in the global epidemiology of pediatric-onset inflammatory bowel disease: systematic review. Gastroenterology. 162: 1147-1159 (2022)

Kwak S, Kim SR, Xu H, Zhang GC, Lane S, Kim H, Jin YS. Enhanced isoprenoid production from xylose by engineered *Saccharomyces cerevisiae*. Biotechnology and Bioengineering. 114: 2581-2591 (2017)

Li MC, He SH. IL-10 and its related cytokines for treatment of inflammatory bowel disease. World Journal of Gastroenterology. 10: 620-625 (2004)

Liu JJ, Kong II, Zhang GC, Jayakody LN, Kim H, Xia PF, Kwak S, Sung BH, Sohn JH, Walukiewicz HE, Rao CV, Jin YS. Metabolic engineering of probiotic *Saccharomyces boulardii*. Applied and Environmental Microbiology. 82: 2280-2287 (2016)

Park JS, Kim YW, Kim H, Kim SK, Park K. Development of a novel ATP bioluminescence assay based on engineered probiotic *Saccharomyces boulardii* expressing firefly luciferase. Journal of Microbiology and Biotechnology. 33: 1506-1512 (2023)

Perli T, van der Vorm DN, Wassink M, van den Broek M, Pronk JT, Daran JM. Engineering heterologous molybdenum-cofactor-biosynthesis and nitrate-assimilation pathways enables nitrate utilization by *Saccharomyces cerevisiae*. Metabolic Engineering. 65: 11-29 (2021)

Ruder B, Atreya R, Becker C. Tumour necrosis factor alpha in intestinal homeostasis and gut related diseases. International Journal of Molecular Sciences. 20: 1887 (2019)

Saijo F, Milsom AB, Bryan NS, Bauer SM, Vowinkel T, Ivanovic M, Andry C, Granger DN, Rodriguez J, Feelisch M. On the dynamics of nitrite, nitrate and other biomarkers of nitric oxide production in inflammatory bowel disease. Nitric Oxide. 22: 155-167 (2010)

Silvestrini L, Rossi B, Gallmetzer A, Mathieu M, Scazzocchio C, Berardi E, Strauss J. Interaction of Yna1 and Yna2 is required for nuclear accumulation and transcriptional activation of the nitrate assimilation pathway in the yeast *Hansenula polymorpha*. PLoS ONE. 10: e0135416 (2015)

Simmonds NJ, Rampton DS. Inflammatory bowel disease - a radical view. Gut. 34: 865-868 (1993)

Siverio JM. Assimilation of nitrate by yeasts. FEMS Microbiology Reviews. 26: 277-284 (2002)

Stanek A, Gadowska-Cicha A, Gawron K, Wielkoszynski T, Adamek B, Cieślar G, Wiczkowski A, Sieroń A. Role of nitric oxide in physiology and pathology of the gastrointestinal tract. Mini-Reviews in Medicinal Chemistry. 8: 1549-1560 (2008)

Szabó C, Ischiropoulos H, Radi R. Peroxynitrite: biochemistry, pathophysiology and development of therapeutics. Nature Reviews Drug Discovery. 6: 662-680 (2007)

Vo PNL, Lee HM, Na D. Synthetic bacteria for therapeutics. Journal of Microbiology and Biotechnology. 29: 845-855 (2019)

Woo SG, Moon SJ, Kim SK, Kim TH, Lim HS, Yeon GH, Sung BH, Lee CH, Lee SG, Hwang JH, Lee DH. A designed whole-cell biosensor for live diagnosis of gut inflammation through nitrate sensing. Biosensors and Bioelectronics. 168: 112523 (2020)