Nitric Oxide Production in Probiotic Lactobacillus Plantarum : Revision of the Origin

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Abstract

Nitric oxide (NO) is formed in gastrointestinal tract by both intestinal mucosa and commensal microbiota. In this work, NO production by probiotic bacteria *Lactobacillus plantarum* 8PA3 was demonstrated by electron paramagnetic resonance (EPR)and NOspecificfluorescent dyes DAF-FM DA (4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate) and DAA (1,2-diaminoanthraquinone sulfate). After we ruled out the generation of NO via denitrification, we showed that NO production by *L. plantarum*depends on L-arginine what is typical for NO-synthases (NOS), and thus proposed NOS-activity in *L. plantarum.* However,we were unable to inhibit the NO production with three specific NOS inhibitorsL-NAME, L-NIL, and nNOS Inhibitor I.We also demonstrated an increased level of NO production in lactobacilli with damaged cellular membranes suggesting stress-dependent NO production by *L. plantarum.*

Keywords: *Lactobacillus plantarum,*denitrification, nitric oxide synthase (NOS), electron paramagnetic resonance (EPR), fluorescent staining.

Introduction

In mammals, nitric oxide (NO) is generated by three isoforms of the enzyme called nitric oxide synthase (NOS) which catalyzes the oxidation of L-arginine to citrulline and NO. In these organisms, NO plays an important role in many biological functions that range from protection against pathogens and tumor cells to blood pressure regulation and nerve transmission (Alderton*et al.,* 2001).

In contrast to eukaryotes, bacteria-derived NO has chiefly been regarded as an intermediate in the nitrogen cycle. In particular during dissimilatory denitrification, nitrite is reduced to NO by nitrite reductase and then further reduced to N_2O and N_2. There are two classes of nitrite reductases, namely those that contain either copper or haem as the cofactor. Neither of these enzymes is structurally or mechanistically similar to the mammalian NOSs (Cutruzzola,1999). Currently, it is known that bacteria, like eukaryotes, can realize L-arginine-dependent NOS-mediated NO production (Crane*et al.,* 2010). The bacterial NOS (bNOS) was first established for a number of bacterial species based

on biochemical detection of NOS activity (Chen& Rosazza,1994, 1995; Choi*et al.,*1997; Sari*et al.,* 1998; Choi*et al.,* 2000; Cohen& Yamasaki2003; Hong*et al.,* 2003). Genome sequencing revealed prokaryotes containing NOS homologs and resulted in cloning of these genes, followed by recombinant expression and characterization of NOS-like proteins: deiNOS (or drNOS) from *Deinococcus radiodurans*(Adak*et al.,* 2002b), saNOS from *Staphylococcus aureus*(Bird*et al.,* 2002; Chartier& Couture, 2004; Salard*et al.,* 2006),bsNOS from *Bacillus subtilis*(Adak et al., 2002a), baNOS from *Bacillus anthracis*(Midha*et al.,* 2005;Salard*et al.,* 2006),gsNOS from *Geobacillus stearothermophilus*(Sudhamsu& Crane,2006), stNOS from *Streptomyces turgidiscabies*(Kers*et al.,* 2004),and scNOS from *Sorangium cellulosum*(Agapie*et al.,* 2009).

Lactobacillus species represent a perspective object to research NO production due to their functional importance in the mammalian intestine as well as their considerable technological and commercial significance (Giraffa*et al.,*2010).Commensal microflora can be a strong source of NO in the human gastrointestinal tract,in particular in the presence of nitrate or nitrite and under anaerobic conditions (Sobko*et al.,*2005, 2006).Due to antimicrobial properties of nitric oxide, NO-producing *Lactobacillus* cells are used in NO-donating therapeutic devices, e.g. probiotic patches (Jones*et al.,* 2010).However, the mechanism of NO synthesisin *Lactobacillus* is still controversial. Nitrite reduction is a rare property of lactic acid bacteria. However, some lactobacilli may reduce nitrate to nitrite and NO under anaerobic conditions (Wolf*et al.,* 1990). Moreover, *L. fermentum* LF1 demonstrated denitrifying nitrite reductase activity under both anaerobic and aerobic conditions (Xu&Verstraete,2001). According to the results of a ^{15}N enrichment experiment, traces of $(NO_2^- + NO_3^-)$-N (total oxidized nitrogen), which seemed to be formed by the resting cells of *L. fermentum* IFO3956, appeared to be derived from L-arginine. Therefore, it was suggested that *L. fermentum* may possess a NOS (Morita*et al.,* 1997). Effects of L-arginine and probiotics on bacterial translocation and extent of liver failure have been studied in a rat acute liver injury model. The results indicated that *L. plantarum* DSM 9843 may containNOS (Adawi *et al.,* 1997), but experiments performed by Morita *et al.*(1997) and Adawi *et al.*(1997) are not enough to prove lactobacillar NO-synthase. Among the most grave drawbacks of the reports cited are that NO production from L-arginine was not investigated in pure culture of *L. plantarum* and the possibility of NO synthesis via denitrification was not assessed.The aim of the present study was toinvestigate if probiotic bacteria *L. plantarum* 8PA3 is able to synthesizeNO and to

further elucidate the origin of detected NO.

Materials and methods

Materials

Sodium diethyldithiocarbamate (DETC) and L-arginine were obtained from Sigma-Aldrich (Germany). Fluorescent NO indicators DAF-FM DA (4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate) and DAA (1,2-diaminoanthraquinone sulfate), and LIVE/DEAD *Bac*Light Bacterial Viability Kit L-7012 were purchased from Molecular Probes (Invitrogen).Inhibitors L-NAME (N[G]-nitro-L-arginine methyl ester, hydrochloride), L-NIL (L-N[6]-(1-imimoethyl)lysine, dihydrochloride), and nNOS Inhibitor I (4S)-N-(4-amino-5[aminoethyl]aminopentyl)-N′-nitroguanidine) were obtained from Calbiochem (Germany).

Bacterial strain and growth conditions

L. plantarum 8PA3 strain was isolated from preparation "Dry lactobacterin" produced by Research and Production Association "Biomed" (Perm, Russia). Unless otherwise specified, *L. plantarum* cultures were grown under microaerobic conditions in de Man-Rogosa-Sharpe (MRS) broth (Merck, Germany) for 48 hoursat 37ºC, and harvested by centrifugation. In experiments investigating the effects of L-arginine and NOS inhibitors on NO production, these substances were dissolved in MRS broth, filter-sterilized and added at the time of inoculation.

NO determination by metmyoglobin method

Plates of MRS agar supplemented with metmyoglobin (MRS-Mb) were prepared as described in (Gundogdu*et al.,* 2006). Bacteria were inoculated onto MRS-Mb by stabbing and were incubated for 2–3 days at 37ºC. NO production was registered by formation of dark red derivatives in medium indicating conversion of metmyoglobin to nitrosomyoglobin.

Determination of stable products of denitrification

The capacity of bacterial culture for denitrification was detected after its growth (37°C, 150 h) under micro-aerobic conditions in MRS broth supplemented with 100 mM KNO_3. Tests were performed with supernatant. Nitrites were quantified with Griess reagent from the photometric

measurement of the absorbance at 540 nm (Green*et al.*, 1982). Nitrates were measured spectrophotometrically with diphenylamine and concentrated sulfuric acid (Bartzatt&Donigan,2004). Gaseous products of denitrification were detected according their accumulation in a float.

EPR spectroscopy

For EPR studies, stationary phase cells were harvested by centrifugation and washed with 50 mM Tris buffer, pH 7.2. The resulting pellet was incubated in the same buffer containing 20 mM L-arginine, or 20 mM KNO_3, and diethyldithiocarbamate-ferrous complex $(DETC)_2$-Fe^{2+} as a spin trap at 37^oC for 1 h. To detect NO resulting from denitrification, the reaction mixture was incubated anaerobically in a CO_2 atmosphere. Control samples did not contain bacteria. EPR spectra were recorded on a Bruker ESP-300 spectrometer (Germany) at liquid nitrogen temperature, microwave power 50 mW, modulation amplitude 1 G. The $(DETC)_2$-Fe^{2+}-NO concentration was determined by double integration of the EPR spectra and comparison with the reference concentration curve which was obtained using solutions with different concentrations of $(DETC)_2$-Cu^{2+} (10^{-3}- 10^{-6} M) in toluene (Iarullina*et al.*,2006).

Fluorescence assays

NO production was assessed with NO sensitive fluorescent dyes: DAF-FM DA, which indicates intracellular NO, and DAA, which can be used to monitor both intra- and extracellular NO. For fluorescent staining, the stationary phase bacteria were harvested by centrifugation and washed three times with sterile Hanks' buffer with calcium and magnesium (PAA Laboratories GmbH, Austria). The resulting pellet was resuspended in the same buffer andincubated for 1 h at 37^oC with 10 mM DAF-FM DA, or 50 ìg/ml DAA. Then cells were washed three times with Hanks' buffer, 5 ml of cell suspension was mounted on glass microscope slides, covered with coverslips, and examined under oil immersion with a fluorescence micro-scope Leica DM6000B (Germany). Fluorescence intensities were measured and analyzed using the Leica FW4000 software.

Viability assay

Growth kinetics were determined at 590 nm on a Lambda 35 double-beam spectrophotometer, Perkin Elmer Instruments (USA). Viability was determined with LIVE/DEAD *Bac*Light bacterial viability kit L-7012, which is based on a mixture of the green

fluorescence nucleic acid stain, SYTO9, that labels all cells in a population, and the red fluorescence nucleic acid stain, propidium iodide (PI), that penetrates only bacteria with damaged membranes and quenches the green stain SYTO9. When used in combination, intact cells are labeled green and cells with damaged membranes are labeled red. Images were obtained with fluorescent microscope Leica DM6000B (Germany) and analyzed using the Leica FW4000 software.

Statistics

The results were processed using Statistica 6.0 software. Three biological replicates were performed for each experiment and the average ± standard deviation was calculated. Student's t-test for paired values was used to determine the significance ($P \leq 0.05$).

Results

Lactobacillus plantarum 8PA3synthesizes NO

First preliminary evidence of NO production in *L. plantarum* 8PA3 came from their capacity to convert metmyoglobin to nitrosomyoglobin. Due to this dark red derivative *L. plantarum* 8PA3 formed good visible rings around their colonies when grown on MRS agar supplemented with metmyoglobin (Fig. 1a). However, this result does not elucidate the origin of detected NO.

To traceNO inside *L. plantarum* 8PA3 cells directly, we took advantageof NO-specific fluorescent dyes DAA and DAF-FM DA. Lactobacilli showed very strong red (in the case of staining with DAA) and green (in the case of staining with DAF-FM DA) fluorescence (Fig. 1b)while the autofluorescence of the cells was below the detection limit (data not shown), indicating presence of NO in bacterial cells. We should note that NO-specific fluorescent dyes also does not clarify the mechanism of NO generation, but provide spatial resolution and possibilities for comparative quantitative evaluation of NO in probes. Surprisingly, bacteria with damaged membranes characterized as dead ones according to staining with PI, showed higher intracellular content of NO compared with intact cells (Fig. 1c), that could be an indicator of stress-induced NO synthesis in *L. plantarum* 8PA3.

NO is not a product of denitrification

Because some *Lactobacillus* species can carry out the dissimilatory nitrate reduction (Wolf*et al.*, 1990;

Xu & Verstraete, 2001), we studied the ability of *L. plantarum* 8PA3 to synthesize NO via denitrification. When incubated micro-aerobically in the medium supplemented with nitrates, bacteria produced trace amounts of nitrites (0.2 - 0.4 ìM) (Table 1), thus indicating nitrate reductase activity of lactobacilli. Gas products of denitrification were not synthesized. It should be noted that NO, as an intermediate of denitrification, cannot be revealed with the method performed, because NO is rapidly transformed in the cells to non-toxic products thus avoiding potential toxicity of the molecule (Goretski *et al.,* 1990). To detect NO possibly formed via denitrification we took advantage of the highly specific NO detection method based on EPR spectroscopy. Bacteria were incubated micro-aerobically with KNO_3 and $(DETC)_2$-Fe^{2+} complex as a spin trap, and NO was quantified. As NO is a highly pervasive hydrophobic molecule that readily penetrates cell walls and membranes (Gusarov*et al.,* 2008), no additional procedures to sonicate cells were performed. Characteristic triplet EPR signal with g-factor close to 2.035 produced by $(DETC)_2$-Fe^{2+}-NO complex was revealed. However, the complex is likely to have abiogenic origin since NO concentrations with and without bacteria did not significantly differ (Fig. 2). As NO can be chemically generated from nitrites at acidic pH (Sobko *et al.,* 2005), a certain level of abiogenic NO could be expected. We therefore conclude that *L. plantarum* 8P-A3 is incapable of NO synthesis via denitrification and suggest that they lack nitrite reductase activity.

NOS substrate L-arginine induces NOproduction

When incubated aerobically with NOS substrate L-arginine *L. plantarum* 8PA3 gave rise to a EPR signal of $(DETC)_2$-Fe^{2+}-NO complex, indicating high amount of NO synthesized by lactobacilli. Notably, NO production in bacteria continued during incubation with $(DETC)_2$-Fe^{2+}. Perhaps, trapping of NO molecule by $(DETC)_2$-Fe^{2+} complex allowed to escape toxic effects of NO, determined mainly by its free radical nature.Since nitrite reductase-derived origin of lactobacillar NO was earlier ruled out, we suggest that *L. plantarum* 8PA3 produce NO through NOS system.

Yet again, NO was present in the cell-free samples, but in considerably smaller amounts than in bacteria-containing samples (Fig. 2). Abiogenic NO output was much higher in anaerobic conditions than in aerobic ones, probably because NO is rapidly oxidized by atmospheric oxygen to form nitrite and nitrate. Registered in cell-free samples under aerobic conditions NO is likely non-enzymatically derived

from L-arginine and H_2O_2 (Nagase*et al.,* 1997). Indeed, some lactobacilli are known to generate hydrogen peroxide (Eschenbach*et al.,*1989).

Growth of *L. plantarum* 8PA3 with 100 ìM L-arginine resulted in a two-fold increase of overall NO production, registered by DAA, whereas intracellular NO content, indicated by DAF-FM DA, did not change (Fig. 1d). We showed that L-arginine has no influence on bacterial viability (Fig. 3a) and growth (Fig. 3b). Thus, the increase in NO production was not due to the effect of the amino acid on cell amount, but was caused by NOS induction.

Inhibitors of eukaryotic NOSs are not effective towards lactobacillar NOS

To evaluate mammalian NOS inhibitors for their effect on NO production by *L. plantarum* 8PA3, bacteria were grown with three NOS inhibitors added into the growth medium in concentrations which were effective towards eukaryotic NOSs. 100 ìM L-NAME, 100 ìM L-NIL, or 10 ìM nNOS Inhibitor I had no effect on NO synthesis in *L. plantarum* 8PA3 as was indicated in fluorescence assays with DAA and DAF-FM DA (Fig 1e). To exclude the difference in viability of inhibitor-treated and control cells, we performed viability assay. The calculated ratio between living (green) and dead (red-orange) cells in allinhibitor-treated variants did not differ from control untreated cells (Fig. 3a) and thus did not depend on the presence of NOS inhibitors. So, we showed that inhibitors of eukaryotic NOSs affect neither bacterial viability, nor NO production.

Discussion

Commensal bacteria can be a significant source of NO in the gut (Sobko*et al.,*2005, 2006), yet the exact mechanism of NO production by intestinal microflora is not clear.Here we show that probiotic *L. plantarum* 8PA3 is able to synthesize NO and provide some evidence for bNOS-derived origin of this metabolite.

Lactobacilli are potentially capable of NO production via denitrification (Wolf*et al.,* 1990; Xu & Verstraete,2001). However, as follows from the accumulation of nitrites in growth medium (Table 1), among all the enzymes of the denitrification pathway only nitrate reductase was detected in *L. plantarum* 8PA3. These results are in agreement with experimental data of Xu and Verstraete (2001), who also found out nitrate reductase activity of lactobacilli. Additionally, this finding was supported
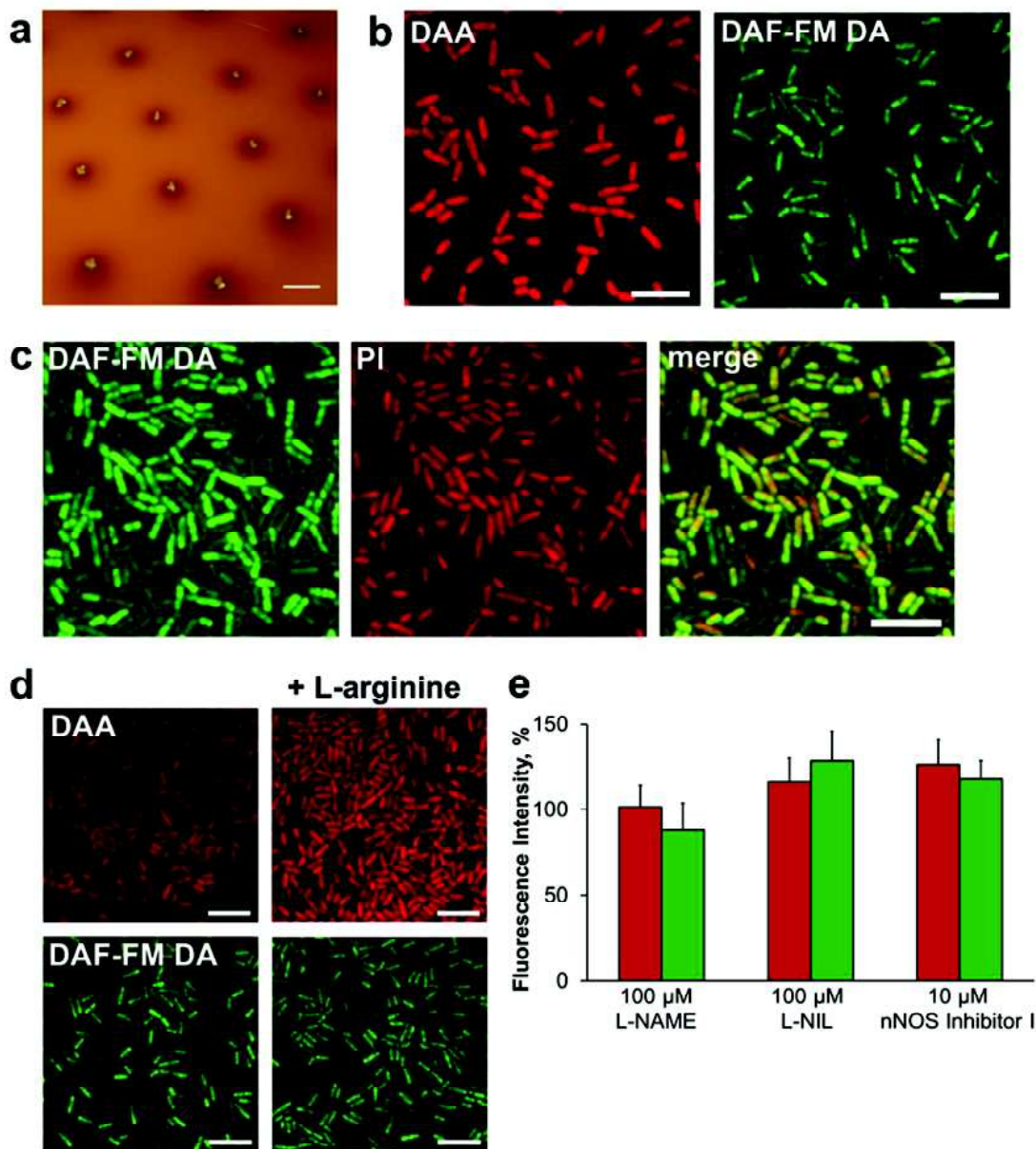
Fig. 1: NOS-mediated production of nitric oxide by *L. plantarum* 8PA3. **a** Formation of nitrosomyoglobin detected by red colored medium around the colonies of *L. plantarum* 8PA3 grown on MRS-Mb. Scale bar = 5 mm.**b** Visualization of NO in *L. plantarum* 8PA3 by fluorescent staining with DAA and DAF-FM DA. **c** Detection of intracellular NO (staining with DAF-FM DA, green color) in bacterial cells with damaged membranes (recognized by staining with PI, red color), presented as a merge image (yellow colored cells). **d** Induction of NO production in lactobacilli by 100 ìM L-arginine added to growth medium. Bacterial cultures in both variants were grown for 48 h and fluorescent stained with DAA or DAF-FM DA. Scale bars (b, c, d) = 5 ìm.**e**NO production in *L. plantarum* 8PA3 grown in the medium with NOS inhibitors measured by fluorescent staining with DAA (red columns) and DAF-FM DA (green columns). Values are expressed as percent of NO level in control cells grown without any additional compounds.
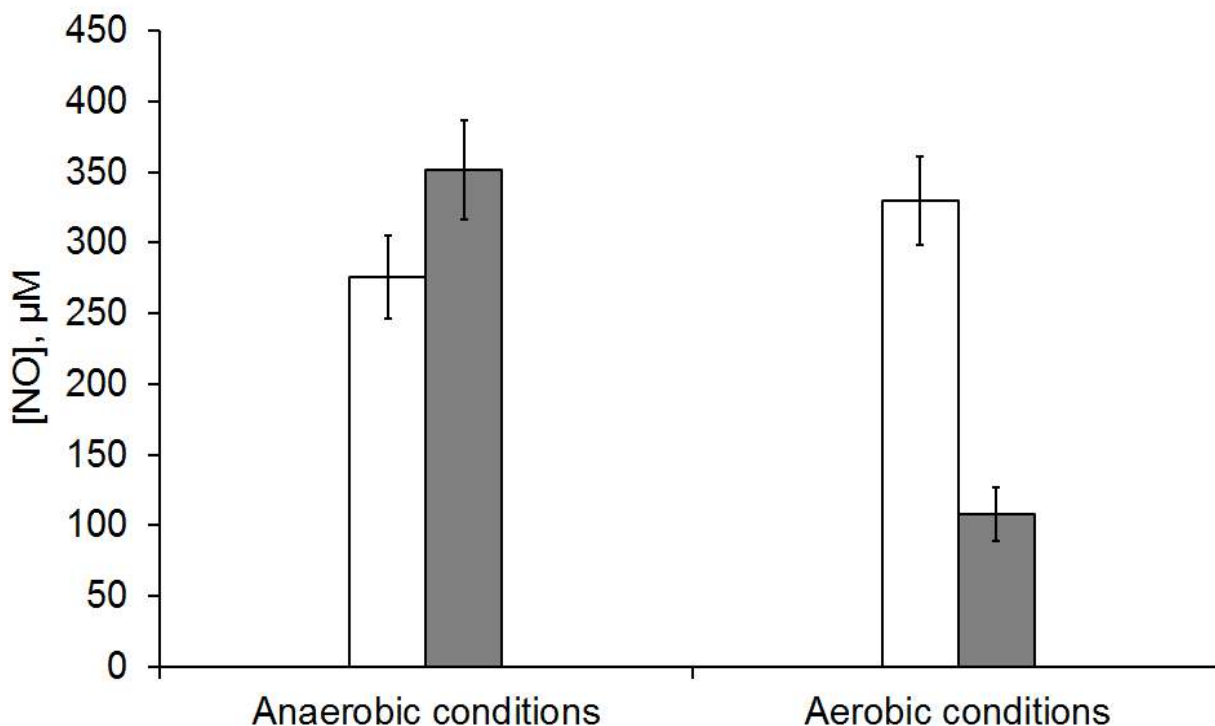
Fig. 2: EPR-measurement of NO content in probes incubated 60 min aerobically with 20 mM L-arginine or anaerobically with 20 mM KNO$_3$ in the presence (blank columns) and absence (filled columns) of *L. plantarum* 8PA3. Each value is the mean of three independent experiments and is expressed as mean ± SD.

Table 1: Detection of denitrification products in *L. plantarum* 8PA3 after 150 h growth in MRS broth supplemented with 100mM [NO$_3^-$]

Strain	Substrate and products of denitrification		
	NO$_3^-$, mM	NO$_2^-$, μM	Gaseous products[*] (N$_2$, N$_2$O), ml^3
L. plantarum 8PA3	50 ± 8	0.3 ± 0.1	0

[*]NO is not accumulated because of the rapid reduction (Goretski *et al.,* 1990).

by our recent genomic screening of *L. plantarum* for homologs of known bacterial nitrate and nitrite reductases. We revealed a cluster of *nar* genes coding for nitrate reductase and no genes for nitrite reductase (Iarullina & Il'inskaia 2007). It is believed, that the predominant pathway for NO production *in vitro* by lactic acid producing lactobacilli is an acidic nonenzymatic reduction of nitrite (Sobko *et al.,* 2005). Using EPR we also detected chemically generated NO in probes incubated anaerobically with nitrate (Fig. 1b).

After we excluded denitrification as a source of NO in *L. plantarum* 8PA3 we further investigated NO production by *L. plantarum* 8PA3 using EPR (Fig. 2) and specific fluorescent NO staining (Fig. 1b). It is likely that under our experimental conditions, NO synthesis could occur through an NO-synthase (NOS)-like activity as described earlier by Adawi *et al. (*1997).For NOS to be responsible for NO production in *L. plantarum* 8PA3, it should have activity characteristic of NO-synthases. Consistently, NOS-mediated bacterial NO production should be suppressed by NOS inhibitors and activated by NOS substrate L-arginine according to L-arginine paradox(Tsikas *et al.,* 2000). As expected, in *L. plantarum* 8PA3 NO production was activated by
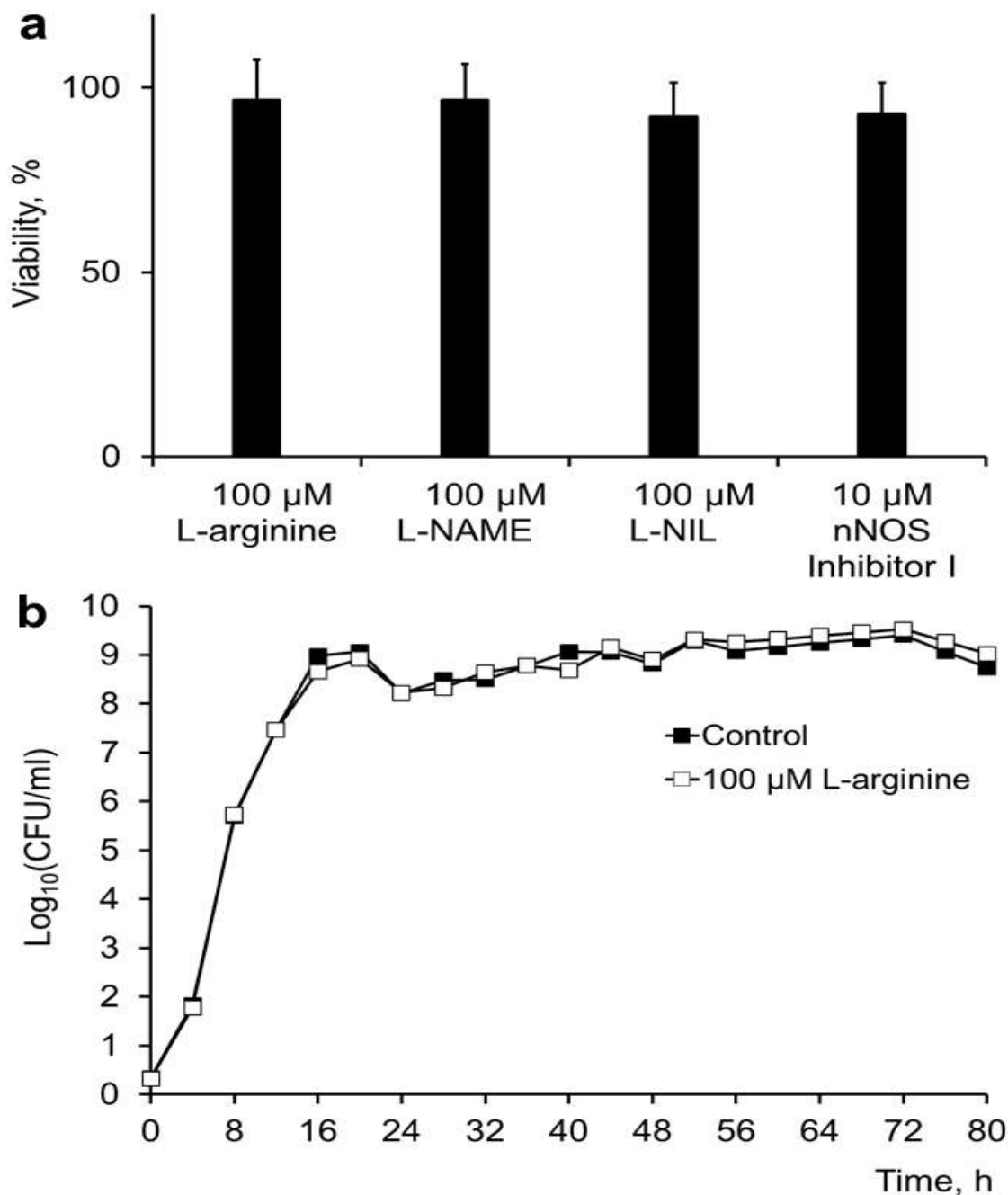
Fig. 3: :The non-effect of L-arginine and NOS inhibitors on *L. plantarum* 8PA3 viability and growth. **a**Viability of *L. plantarum* 8PA3 (48 h, MRS medium) in the presence of L-arginine and NOS inhibitors according to staining with LIVE/DEAD *Bac*Light Bacterial Viability Kit. Values are expressed as percentage viability of control cells grown without any additional compounds. **b** Time course of *L. plantarum* 8PA3 growth in the presence (open squares) and absence (filled squares) of 100 ìM L-arginine. Each value is the mean of three independent experiments. SD≤8%.

exogenous L-arginine (Fig. 1d) thus suggesting bNOS-mediated NO production. We used inhibitors with different selectivity for the three human NOS isoforms: L-NIL is more selective for the inducible NOS, nNOS Inhibitor I – for neuronal NOS, and L-NAME is slightly more selective for endothelial NOS, but none of these NOS inhibitors was efficient towards NO production by *L. plantarum* 8PA3 (Fig. 1e). These results are probably caused by degradation of the inhibitors by bacteria or membrane impermeability for them. For example, the inhibitors of mammalian NOSs inhibited the activity of bacterial NOSs in crude homogenates or purified preparations, but not in intact *Staphylococcus aureus* (Choi *et al.,* 1998) and *Rhodococcus* sp. R312 cells (Cohen & Yamasaki, 2003). It should be noted that currently it is difficult to assign detected NO synthesis in *L. plantarum* 8PA3 to the certain NOS homolog. In genome of *L. plantarum* we revealed the flavodoxin protein with high homology to the C-terminal reductase domain of eukaryotic NOS – essential redox partners of the catalytic NOS oxygenase domain (NOS_{ox}) (Iarullina and Il'inskaia 2007). Nevertheless, none of the *Lactobacillus* species sequenced contain the NOS_{ox} homolog (Crane *et al.,* 2010). Probably, unefficiency of NOS inhibitors against NO synthesis in *L. plantarum* 8PA3 results from significant differences in structure of lactobacillar NOS and known NOSs. Further biochemistry and genetics studies of *L. plantarum* must clarify protein and gene determinants of detected NO production.

Fluorescent staining with DAA and DAF-FM DA demonstrated NO presence in almost all cells of the population. This finding points to the significance of NO function inside bacteria. Interestingly, lactobacilli were labeled with DAF-FM DA to a different extent, indicating non-equal NO content in different cells of the culture (Fig. 1b). It is well-established that cells in non-synchronized culture are in different stages of cell-cycle. Using the red fluorescence nucleic acid stain PI we revealed an increased level of NO production in lactobacilli with damaged cellular membranes (Fig. 1c), that could be due to the stress-dependent NO production. Earlier it was already shown that bNOS protects *Bacillus subtilis* and *B. anthracis* against oxidative stress (Gusarov & Nudler, 2005; Shatalin *et al.,* 2008). Because NO is a potent cytotoxic agent, it may be suggested that NO itself may cause cell damage. However, activation of NO synthesis by L-arginine did not affect bacterial growth and viability (Fig. 3). When lactobacilli were grown with L-arginine overall NO production increased, while intracellular NO content stayed at a basal level (Fig. 1d). It is known

that NO molecules have high permeability through the membranes of cells due to their small sizes and the absence of charge (Gusarov *et al.,* 2008). Currently, we can only speculate how lactobacilli keep a steady-state concentration of potentially pervasive and toxic NO inside their cells. Probably, they possess an adaptive system which regulates NO concentration and is important for the viability of bacteria in their natural environment in the mammalian intestine.

In summary, we demonstrated L-arginine dependent aerobic NO production by *L. plantarum* 8PA3, thus including the strain to a cohort of Gram-positive NOS-containing bacteria. The discovery of NO producing pathway in commensal probiotic bacteria similar to the one performed in surrounding intestinal mucosa is rather promising. Practical application of bNOS-containing probiotic bacteria offers a novel approach for a regulated and continuous delivery of physiological amounts of NO for research and medical purposes. As the same molecule appears to serve different masters, the interplay between probiotic- and host-derived NO is particularly engaging. In this context, an important problem is the role of bacteria-derived NO in both producers and eukaryotes. Our results indicate NO association with stress-response in *L. plantarum* 8PA3. Whether there is any connection between NO production and probiotic activity of *Lactobacillus* species are still essential questions to be addressed. It is quite likely that NO formation by probiotic bacteria contributes to their health promoting effects on the organism, mechanisms of which are not entirely understood yet.

References

1. Adak S, Aulak KS, Stuehr DJ (2002a) Direct evidence for nitric oxide production by a nitric-oxide synthase-like protein from *Bacillus subtilis*. J Biol Chem 277(18):16167-16171

2. Adak S, Bilwes AM, Panda K, Hosfield D, Aulak KS, McDonald JF, Tainer JA, Getzoff ED, Crane BR, Stuehr DJ (2002b) Cloning, expression and

characterization of a nitric oxide synthase protein from *Deinococcus radiodurans*. Proc Natl Acad Sci USA 99(1):107-112

3. Adawi D, Kasravi FB, Molin G, Jeppsson B (1997) Effect of *Lactobacillus* supplementation with and without arginine on liver damage and bacterial translocation in an acute liver injury model in the rat. Hepatology 25(3):642-647

4. Agapie T, Suseno S, Woodward JJ, Stoll S, Britt RD, Marletta MA (2009) NO formation by a catalytically self-sufficient bacterial nitric oxide synthase from *Sorangium cellulosum*. Proc Natl Acad Sci USA 106(38):16221-16226

5. Alderton WK, Cooper CE, Knowles RG (2001) Nitric oxide synthases: structure, function and inhibition. Biochem J 357:593–615

6. Bartzatt R, Donigan L (2004) The colorimetric determination of nitrate anion in aqueous and solid samples utilizing an aromatic derivative in acidic solvent. Toxicol Environ Chem 86:75-85

7. Bird LE, Ren J, Zhang J, Foxwell N, Hawkins AR, Charles IG, Stammers DK (2002) Cryctal structure of SANOS, a bacterial nitric oxide synthase oxygenase protein from *Staphylococcus aureus*. Structure 10(12):1687-1696

8. Chartier FJM, Couture M (2004) Stability of the heme environment of the nitric oxide synthase from *Staphylococcus aureus* in the absence of pterin cofactor. Biophys J 87(3):1939-1950

9. Chen Y, Rosazza JPN (1994) A bacterial nitric oxide synthase from a *Nocardia* species. Biochem Biophys Res Commun 203(2):1251-1258

10. Chen Y, Rosazza JPN (1995) Purification and characterization of nitric oxide synthase (NOS_{NOC}) from a *Nocardia* species. J Bacteriol 177(17):5122-5128

11. Choi DW, Oh HY, Hong SY, Han JW, Lee HW (2000) Identification and characterization of nitric oxide synthase in *Salmonella typhimurium*. Arch Pharm Res 23(4):407-412

12. Choi WS, Chang MS, Han JW, Hong SY, Lee HW (1997) Identification of nitric oxide synthase in *Staphylococcus aureus*. Biochem Biophys Res Commun 237(3):554-558

13. Choi WS, Seo DW, Chang MS, Han JW, Hong SY, Paik WK, Lee HW (1998) Methylesters of L-arginine and N-nitro-L-arginine induced nitric oxide synthase in *Staphylococcus aureus*. Biochem Biophys Res Commun 246(2):431-435

14. Cohen MF, Yamasaki H (2003) Involvement of nitric oxide synthase in sucrose-enhanced hydrogen peroxide tolerance of *Rhodococcus* sp. strain APG1, a plant-colonizing bacterium. Nitric Oxide 9(1):1-9

15. Crane BR, Sudhamsu J, Patel BA (2010) Bacterial nitric oxide synthases. Annu Rev Biochem 79:445-470

16. Cutruzzola F (1999) Bacterial nitric oxide synthesis. Biochim Biophys Acta 1411(2-3):231-249

17. Eschenbach DA, Davick PR, Williams BL, Klebanoff SJ, Young-Smith K, Critchlow CM, Holmes KK (1989) Prevalence of hydrogen peroxide-producing *Lactobacillus* species in normal women and women with bacterial vaginosis. J Clin Microbiol 27(2):251-256

18. Giraffa G, Chanishvili N, Widyastuti Y (2010) Importance of lactobacilli in food and feed biotechnology. Res Microbiol 161(6):480-487

19. Goretski J, Zafiriou OC, Hollocher TC (1990) Steady-state nitric oxide concentrations during denitrification. J Biol Chem 265(20):11535-11538

20. Green L, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR (1982) Analysis of nitrate, nitrite, an [^{15}N]nitrate in biological fluids. Anal Biochem 126(1):131-138

21. Gundogdu AK, Karahan AG, Cakmakc ML (2006) Production of nitric oxide (NO) by lactic acid bacteria isolated from fermented products. Eur Food Res Technol 223:35-38

22. Gusarov I, Nudler E (2005) NO-mediated cytoprotection: instant adaptation to oxidative stress in bacteria. Proc Natl Acad Sci USA 102(39):13855–13560

23. Gusarov I, Starodubtseva M, Wang Z-Q, McQuade L, Lippard SJ, Stuehr DJ, Nudler E (2008) Bacterial nitric-oxide synthases operate without a dedicated redox partner. J Biol Chem 283(19):13140-13147

24. Hong I, Kim YK, Choi WS, Seo DW, Yoon JW, Han J-W, Lee HY, Lee HW (2003) Purification and characterization of nitric oxide synthase from *Staphylococcus aureus*. FEMS Microbiol Lett 222(2):177-182

25. Iarullina DR, Il'inskaia ON (2007) Genomic determinants of nitric oxide biosynthesis in *Lactobacillus plantarum*: potential opportunities and reality. Mol Biol (Mosk) 41(5):900-907

26. Iarullina DR, Il'inskaia ON, Aganov AV, Silkin NI, Zverev DG (2006) Alternative pathways of

nitric oxide formation in lactobacilli: EPR evidence for nitric oxide synthase activity.Mikrobiologiia 75(6):731-736

27. Jones ML, Ganopolsky JG, Labbe A, Prakash S (2010) A novel nitric oxide producing probiotic patch and its antimicrobial efficacy: preparation and *in vitro* analysis. Appl Microbiol Biotechnol 87(2):509-516

28. Kers JA, Wach MJ, Krasnoff SB, Widom J, Cameron KD, Bukhalid RA, Gibson DM, Crane BR, Loria R (2004) Nitration of a peptide phytotoxin by bacterial nitric oxide synthase. Nature 429:79-82

29. Midha S, Mishra R, Aziz MA, Sharma M, Mishra A, Khandelwal P,Bhatnagar R (2005) Cloning, expression and characterization of recombinant nitric oxide synthase-like protein from *Bacillus anthracis*. Biochem Biophys Res Commun 336(1):346-356

30. Morita H, Yoshikawa H, Sakata R, Nagata Y, Tanaka H (1997) Synthesis of nitric oxide from the two equivalent guanidino nitrogens of L-arginine by *Lactobacillus fermentum*. J Bacteriol 179(24):7812-7815

31. Nagase S, Takemura K, Ueda A, Hirayama A, Aoyagi K, Kondoh M, Koyama A (1997) A novel nonenzymatic pathway for the generation of nitric oxide by the reaction of hydrogen peroxide and D- or L-arginine. Biochem Biophys Res Commun 233(1):150-153

32. Salard I, Mercey E, Rekka E, Boucher J-L, Nioche P, Mikula I, Martasek P, Raman CS, Mansuy D (2006) Analogies and surprising differences between recombinant nitric oxide synthase-like proteins from *Staphylococcus aureus* and *Bacillus anthracis* in their interactions with L-arginine analogs and iron ligands. J Inorg Biochem 100(12):2024-2033

33. Sari M-A, Moali C, Boucher J-L, Jaouen M, Mansuy D (1998) Detection of a nitric oxide synthase possibly involved in the regulation of the *Rhodococcus* sp. R312 nitrile hydratase. Biochem Biophys Res Commun 250(2):64-368

34. Shatalin K, Gusarov I, Avetissova E, Shatalina Y, McQuade LE, Lippard SJ, Nudler E (2008) *Bacillus anthracis*-derived nitric oxide is essential for pathogen virulence and survival in macrophages. Proc Natl Acad Sci USA 105(3):1009–1013

35. Sobko T, Huang L, Midtvedt T, Norin E, Gustafsson LE, Norman M, Jansson EA, Lundberg JO (2006) Generation of NO by probiotic bacteria in the gastrointestinal tract. Free Radic Biol Med 41(6):985-991

36. Sobko T, Reinders CI, JanssonEA, NorinE, MidtvedtT,LundbergJO (2005) Gastrointestinal bacteria generate nitricoxide from nitrate and nitrite. Nitric Oxide 13(4):272–278

37. Sudhamsu J, Crane BR (2006) Structure and reactivity of a thermostable prokaryotic nitric-oxide synthase that forms a long-lived oxy-heme complex. J Biol Chem 281(14):9623-9632

38. Tsikas D, Boger RH, Sandmann J, Bode-Boger SM, Frolich JC (2000) Endogenous nitric oxide synthase inhibitors are responsible for the L-arginine paradox. FEBS Lett 478(1-2):1-3

39. Wolf G, Arendt EK, Pfähler U, Hammes WP (1990) Heme-dependent and heme-independent nitrite reduction by lactic acid bacteria results in different N-containing products. Internat J Food Microbiol 10(3-4):323-330

40. Xu J, Verstraete W (2001) Evaluation of nitric oxide production by lactobacilli. Appl Microbiol Biotechnol 56(3-4):504-507