Supplementary Material

H_2O_2 production by lactobacilli promotes epithelial restitution during colitis

Ashish K. Singh[a], Rosanne Y. Hertzberger[b,c,¥], Ulla G. Knaus[a,§]

[a] Conway Institute, School of Medicine, University College Dublin, Dublin, Ireland
[b] Swammerdam Institute for Life Sciences, University of Amsterdam, Amsterdam and [c] NIZO Food Research, Ede, Netherlands

This file contains:

A. Supplementary Methods

B. Supplementary Tables 1-3

C. Supplementary References

D. Figures: Figure S1, Figure S2

A. Supplementary Methods

Construction of *L. johnsonii* double deletion mutant (DEL)

The construction of the *L. johnsonii* single deletion derivatives Δ*LJ_1254-LJ_1255* (*Δnox*, NCC9337) and Δ*LJ_0548-LJ_0549* (*Δnfr*, NCC9359) were described in [1]. An overview of all plasmids used in this study is presented in Table S2. For this study a double deletion mutant that lacks both *LJ_1254-LJ_1255 (nox)* and *LJ_0548-LJ_0549* (*nfr*) was constructed. To obtain the double deletion mutant, the erythromycin cassette was removed from NCC9359 (Δ*nfr*) by the plasmid-borne expression of the *Saccharomyces cerevisiae flp* gene (flp_{SC}) using pDP893. The construction of pDP893 employed *Lactococcus lactis* MG1363 as an intermediate cloning host. It was constructed by amplifying the *pgiA* promoter (P*pgiA*) using *Lactococcus lactis* MG1363 genomic DNA as a template with the primers A and B (Table S3); the resulting amplicon was digested with *Spe*I and *Hin*dIII and ligated in similarly digested pDP600 (Cm^r, Em^r) [2] generating pDP600-P*pgiA*. The predicted bi-directional terminator positioned between the *LJ_1125* and *LJ_1126* genes of NCC 533 (T*LJ1125*) was amplified using genomic DNA of this *L. johnsonii* strain as a template and the primers C and D (Table S3). The resulting amplicon was digested with *Hin*dIII and *Xho*I and cloned into similarly digested pDP600-P*pgiA*, to yield pDP600-P*pgiA*-T*LJ1125*. The *Saccharomyces cerevisiae* SC288c 2 micron plasmid [3] was isolated and digested with *Sph*I and *Xba*I, and the 1.46 kb fragment containing the *flp* gene was isolated and cloned in similarly digested pDP600-P*pgiA*-T*LJ_1125*, yielding plasmid pDP893. This plasmid encodes the *flp* gene under transcriptional control of the *pgi*A promoter and is followed by the T*LJ1125* terminator.

Plasmid pDP893 was isolated from *Lactococcus lactis* MG1363 and used to transform NCC9359. Transformants were cultured in MRS medium supplemented with chloramphenicol at 32°C for 5 serial passages (permissive temperature for plasmid replication), and then plated to isolate single, chloramphenicol resistant colonies, which were subsequently replica plated to confirm erythromycin sensitivity. The selected colony with the required antibiotic resistance phenotype (Cm^r, Em^r) was cultured in MRS at 37°C for three serial passages, and subsequently plated to identify single colonies that were chloramphenicol and erythromycin sensitive by replica plating on plates containing these antibiotics. The selected strain was designated NCC9359-FO (FLP-out). NCC9359-FO was transformed with pDP902 to achieve the deletion of the *nox* locus as described earlier [1]. The deletion was

confirmed using primers flanking the target regions. The *Δnfr Δnox* derivative of *L. johnsonii* NCC533 was designated NCC9360. In this study for the purpose of simplification, double deletion mutant NCC9360 was designated DEL.

B. Supplementary Tables: Table S1

Disease Activity Index (DAI)		
Parameter		***Score***
Body weight loss (%)	0 %	0
	< 3%	1
	3-6%	2
	6-10%	3
	>10%	4
Fecal consistency	normal	0
	Shaped soft	2
	Unshaped, diarrhea	
Presence of blood	None	0
	Visible	2
	Frank blood	4
Clinical scores		
Parameter		***Score***
Feces	Normal	0
	Soft and shaped	1
	Soft unshaped/ with blood/ diarrhea without blood	2
	Dysentery/ frank blood	3
Body weight loss (%)	Normal	0
	<10%	1
	10-20%	2
	>20%	3
Behaviour	Normal	0
	Minor depression/ exaggerated response	1
	Decreased mobility or alertness/ isolated	2
	Still/ pre-comatose	3
Appearance	Normal	0
	Mild piloerection/ mild dehydration	1
	Dehydration/ hunched/ piloerection	2
	Very thin/ severe dehydration	3

Table S2: An overview of strains and plasmid used in this study

Strains and plasmids	Genotype	Reference
NCC533	Wild type *L. johnsonii*	[4]
NCC9337	Em^R, *Δnox* (*LJ_1254-LJ_1255*), predicted to encode NADH oxidase	[1]
NCC9359	Em^R, *Δnfr* (*LJ0548-LJ0549*) encoding NADH flavin reductase	[1]
NCC9360	Em^R, *Δnox, Δnfr* (Δ*LJ_1254-LJ_1255*, Δ*LJ_0548-LJ_0549*)	This study
pDP600	a chloramphenicol resistant version of pG+host9 containing a complete pBluescript array of unique restriction sites	[2]
pDP893	pDP600- P*pgiA* - flp_{SC} - *LJ_1125* trm	This study

Table S3: Primers, strains and plasmids used to construct double deletion mutant DEL (*L. johnsonii* NCC9360)

Primers	Sequence
A	ATATATACTAGTACCCTTAAAAGTGTTAGGAG
B	ATATATAAGCTTGAGCTCGCTAGCGCATGCTAATTCCTTTCAATTTCTCGC
C	ATATATAAGCTTTGCCAATGGATAACCAGG
D	ATATATCTCGAGAATCTCTCTTGGACTTGC
E	ATATTGGATCCCCAGTTGATGAAGTTTTGAAATTCG
F	CATAAGAATTCCACCATGTTTAAAAGTTACTTTGTCGG

C. Supplementary References:

[1] R. Hertzberger, J. Arents, H.L. Dekker, R.D. Pridmore, C. Gysler, M. Kleerebezem, M.J.T. de Mattos, H(2)O(2) production in species of the Lactobacillus acidophilus group: a central role for a novel NADH-dependent flavin reductase., Appl. Environ. Microbiol. 80 (2014) 2229–39. doi:10.1128/AEM.04272-13.

[2] E. Denou, R.D. Pridmore, B. Berger, J.M. Panoff, F. Arigoni, H. Brüssow, Identification of genes associated with the long-gut-persistence phenotype of the probiotic Lactobacillus johnsonii strain NCC533 using a combination of genomics and transcriptome analysis, J. Bacteriol. 190 (2008) 3161–3168. doi:10.1128/JB.01637-07.

[3] J.L. Hartley, J.E. Donelson, Nucleotide sequence of the yeast plasmid, Nature. 286 (1980) 860–864. doi:10.1038/286860a0.

[4] R.D. Pridmore, B. Berger, F. Desiere, D. Vilanova, C. Barretto, A.-C. Pittet, M.-C. Zwahlen, M. Rouvet, E. Altermann, R. Barrangou, B. Mollet, A. Mercenier, T. Klaenhammer, F. Arigoni, M.A. Schell, The genome sequence of the probiotic intestinal bacterium Lactobacillus johnsonii NCC 533, Proc. Natl. Acad. Sci. 101 (2004) 2512–2517. doi:10.1073/pnas.0307327101.

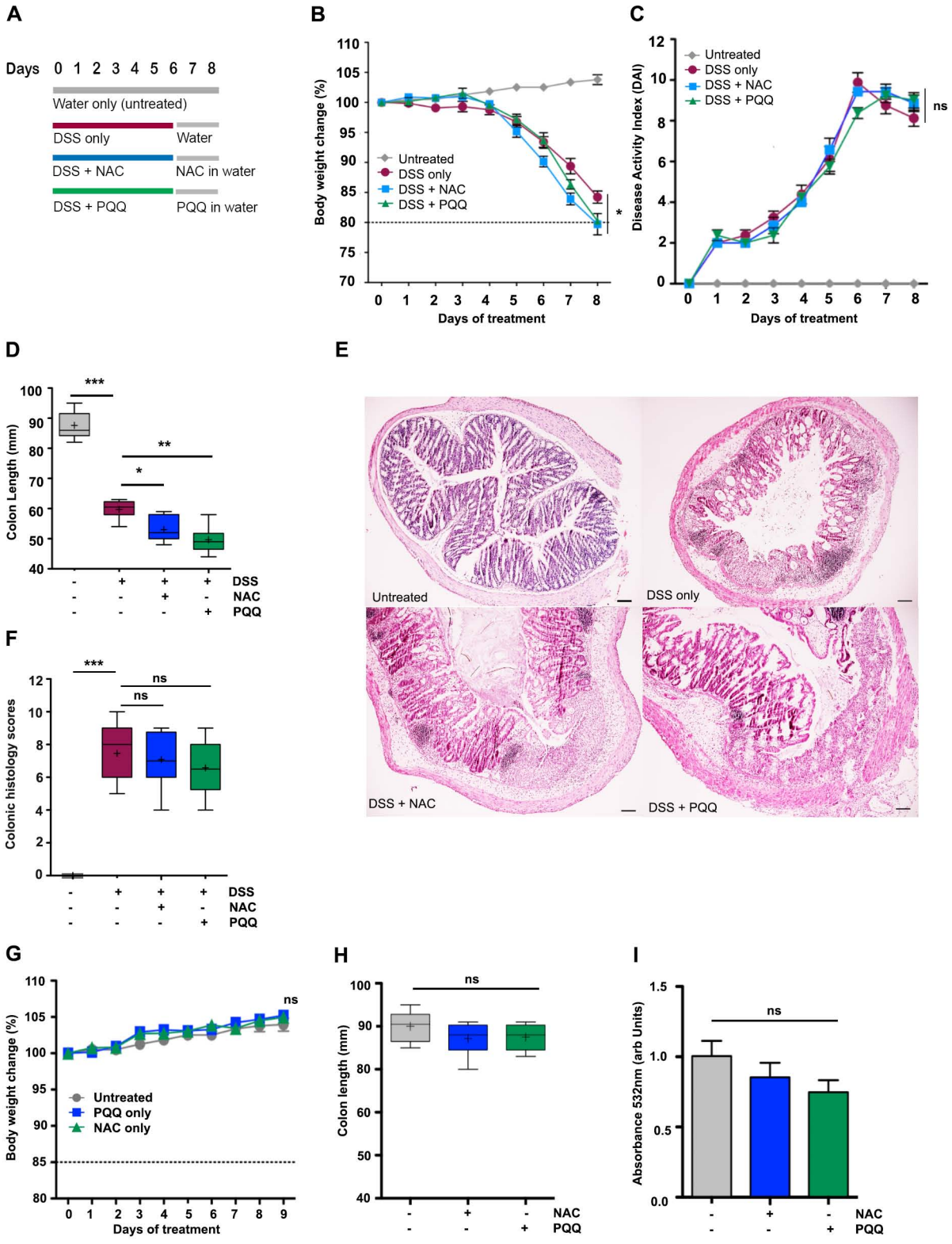

Fig. S1. High dose of oral antioxidants is not protective in DSS colitis. Mice (n=8) were treated with or without DSS in the presence or absence of indicated antioxidants. (A) Schematic representation of experimental groups and treatments (NAC, PQQ) for (A-F). (B) Body weight profile, (C) disease scores, (D) colon length at day 8, (E) colonic sections stained with H&E (scale bar 100µm), (F) histology scores. (G-I) Analysis of antioxidant effects without DSS treatment. (G) Body weight, (H) colon length and (I) lipid peroxidation of mice exposed to sterile water with or without added NAC or PQQ for 9 days. (B, C, G) are represented as mean ±SEM, (D, F, H) as mean Whiskers (Min and Max) with "+" denoting mean point. Significance was determined with one-way ANOVA (B, D, G, H, I) and Mann-Whitney non-parametric test (C, F). *p<0.05, **p<0.01, ***p<0.001, non-significant (ns).

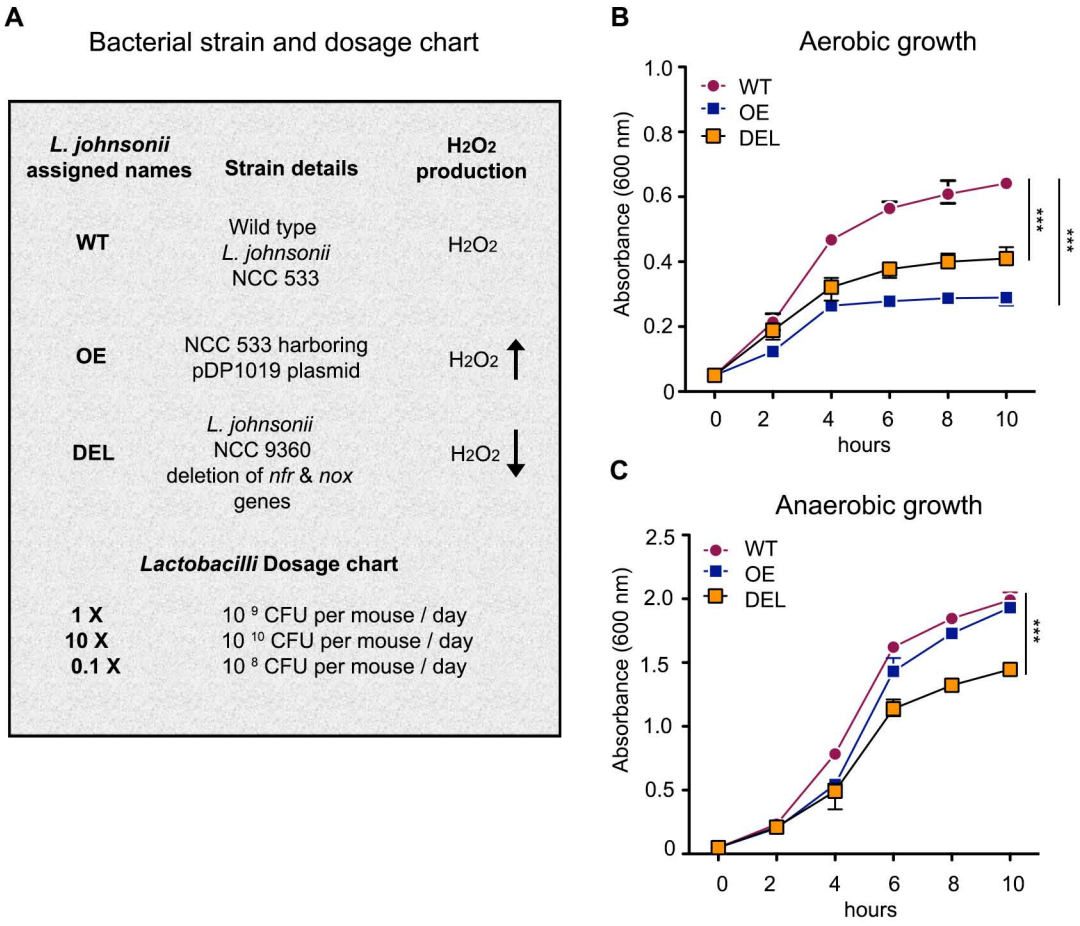

Fig. S2. Bacterial strain designations and growth curves of *L. johnsonii* strains (n=3). (A) *L. johnsonii* strains used in this study and their characteristics. *L. johnsonii* dosage chart indicates bacterial doses gavaged per mouse each day. (B, C) Growth curves of *L. johnsonii* strains in aerobic and anaerobic conditions respectively. All values are represented as mean ± SD and were analyzed by one-way ANOVA. ***p<0.001.