Gut bacterial tyrosine decarboxylases restrict levels of levodopa in the treatment of Parkinson's disease

van Kessel et al.

Supplementary methods

Screening for *Enterococcus* **strains, isolated from healthy subjects and clinical isolates** *Enterococcus* strains were isolated from fecal or urine samples from clinical setting or from healthy volunteers (aged 2 to 79 years). All samples were collected between January 2008 and 2018 in Beni-Suef City, Egypt. A total of 77 *Enterococcus* spp. were isolated on bile esculin agar and observed microscopically after gram staining. The screening for decarboxylase activity was performed as described previously by Bover-Cid and Holzapfel¹ with levodopa or tyrosine added as a substrate to a final concentration of 1% to screen for production of dopamine and tyramine. All *Enterococcus* strains were spot inoculated on agar plates containing the substrates of interest and on control plates without any substrate. The plates were duplicated, either aerobically or anaerobically at 37°C. Plates were checked daily for 4 days to record the change in the indicator color from yellow to violet, indicative of production of tyramine and dopamine, respectively (**Supplementary Table 1**).

Fecal samples from patients with Parkinson's disease

All study subjects consented to the use of their samples for research. Parkinson's disease was diagnosed according to the UK Brain Bank Criteria. Exclusion criteria for Parkinson's disease subjects: (1) atypical or secondary Parkinsonism, (2) the use of probiotics or antibiotics within three months prior to sample collection, (3) primary gastrointestinal pathology, (4) unstable medical, neurological, or psychiatric illness, (5) low platelet count (<80k), uncorrectable prolonged PT (>15 sec. Solid fecal samples were collected via a home feces collection kit. Study patients were provided with the supplies and instructions for home feces collection using the BD Gaspak EZ Anaerobe Gas Generating Pouch System with Indicator (Ref 260683; Becton, Dickinson and Company, Sparks, MD) in order to minimize the exposure of the feces to high oxygen ambient atmosphere, which may alter the microbiota. Patients were asked to have a bowel movement within 24 hours of their study visit. Patients kept the sealed anaerobic fecal bag in a cold environment, before bringing the anaerobic fecal bag to the hospital. Fecal samples were then immediately stored at -80°C until analysis.

Primers targeting bacterial tyrosine decarboxylase

In order to cover numerous bacterial species harboring the *tdc* gene, degenerate primers, Dec5f and Dec3r, previously designed to target 350bp region of the *tdc* gene from a variety of bacterial genera² were used for qPCR. Prior to qPCR experiments a normal PCR test on the genomic DNA *E. faecalis* v583 was performed using Phire Hot Start II DNA Polymerase (F125S, ThermoFisher) using the following PCR parameters: 3 min at 98°C; 15 sec at 98°C, 1 min at 58°C and 1 min at 72°C, for 35 cycles; 5 min at 72°C, to ensure primer specificity.



Supplementary Figure 1. Corresponding control samples jejunal content incubation without levodopa. From left to right corresponding control samples, where H₂O was added instead of L-DOPA. Bacterial conversion of tyrosine (TYR) to tyramine (TYRM) during 24 hrs of incubation of jejunal content is visible. No dopamine is produced *de novo* or hydrolyzed from potential conjugated-forms of dopamine in the jejunal-content.



Supplementary Figure 2. Microbiota harboring other PLP-dependent amino acid decarboxylases do not decarboxylate levodopa. (a) Phylogenetic analysis of TDC proteins from NIH Human Microbiome Project (HMP) protein database, TDC_{EFS} (EOT87933) was used as query. TDC protein sequences from strains employed in this study are depicted in bold. Live stationary cultures of *L. brevis* grown with levodopa in (b) MRS (De Man, Rogosa and Sharpe) or in (c) enriched beef broth (EBB) buffered at pH 6.0. (d-g) Gut associated bacteria harboring different amino acid decarboxylases, which

were previously identified (**Supplementary Table 2**) were tested for their ability to convert levodopa in live stationary cultures



Supplementary Figure 3. IC50 determination for human DOPA decarboxylase and bacterial tyrosine decarboxylases. (a) Kinetic curve with levodopa as substrate for human DOPA decarboxylase (DDC) to determine the inhibitory constant for carbidopa. Reactions were performed in triplicate using 10 nM of enzyme in 100 mM PO₄ with 100 μ M PLP³ with levodopa concentrations ranging from 0.1-1.0 mM. The enzyme kinetic parameters were calculated using nonlinear Michaelis-Menten regression model (for further kinetic parameters see **Table 1**). IC50 inhibitory curves using carbidopa as inhibitor for (b) DDC (0.005-2.56 μ M carbidopa), (C) TDC_{EFS} (2-1024 μ M carbidopa), (d) TDC_{EFM} (2-1024 μ M carbidopa), (e) ^PTDC_{EFM} (2-1024 μ M carbidiopa). Reactions were performed in triplicate and the parameters were determined by fitting a sigmoidal–curve ([inhibitor] vs. normalized response). Further parameters are listed in **Supplementary Table 3**.



Supplementary Figure 4. Human DOPA decarboxylase inhibitors are ineffective against the decarboxylase activity of live enterococci. Live stationary cultures of *E. faecalis* and *E. faecium* incubated for 15 minutes with (a) an equimolar ratio levodopa /carbidopa, (b, c) a 6:1 molar ratio (4:1 in weight) of levodopa /benserazide or 4.8:1 molar ratio (4:1 in weight) of levodopa/methyldopa (100/16.7/20,8 μ M levodopa /benserazide/methyldopa). Samples were analyzed using HPLC-ED. Bar graphs show levels of dopamine production (relative to control, where no inhibitor was added) with and

without the addition of inhibitor. Error bars represent the SEM and significance was tested using a parametric unpaired T-test (*=p<0.02).



Supplementary Figure 5. Primers (Dec5f and Dec3r) targeting *E. faecalis* v583 *tdc* gene. (a) Target

of the Dec5f and Dec3r primers² are depicted for *Enterococcus faecalis* v583 with (**b**) the corresponding

agarose gel of PCR amplification of 336 bp fragment of tdc.

Supplementary Figure 6. Identification of conserved *tdc* paralogue protein (TDC_{EFM}) in all *E*.

faecium strains analyzed. Identification of conserved *tdc* paralogue protein (TDC_{EFM}) in all *E. faecium* strains analyzed. Genome contigs harboring the *tdc* gene cluster of all *E. faecium* strains were extracted from NCBI and aligned using Mauve genome aligner. As comparison, the genome of *E. faecium* W54 is depicted above the alignment results. The paralog TDC from *E faecium* ($^{P}TDC_{EFM}$) is shown in orange. Black boxes indicate the TDC gene in all other strains, white bars indicate the single genes, and colored bars indicate conserved gene clusters. Due to its size, this figure is merged at the end of the supplementary material document.

Supplementary Table 1. Enterococcus strains isolated from healthy subjects and clinical

	Number	Sample	Гуре	Bile aesculin hydrolysis	Levodopa decarboxylation	Tyrosine decarboxylation
	29	Clinical	Stool	29	29	29
	34	Clinical	Urine	34	33	33
	14	Volunteer	Stool	14	10	10
Total	77			77	72	72

enterococcal isolates

Enterococcus strains isolated from (1) fecal samples (collected from non-hospitalized subjects from clinical labs during their routine check up), (2) urine samples (collected from non-hospitalized patients suffering from urinary tract infection), and (3) healthy volunteers (age 2 to 79 years). All samples were isolated between January 2008-2018 in Beni-Suef City, Egypt. 72 out of 77 isolates were able to decarboxylate levodopa and tyrosine indicating that only 5 isolates are species or strains not encoding for tyrosine decarboxylase.

Reported tyrosine decarboxylation ⁴	Cloned gene ⁴	Associated protein accession	Definition	This study (Live bacterial culture)	Protein Identity*	Protein accession
-	BF 0393 (BF9343_0382)	CAH06161	Putative Glutamate decarboxylase	Bacteroides fragilis NCTC 9343 (=ATCC 25285)	100.00%	CAH06161
	Not tested	NA	Tyrosine decarboxylase	Enterococcus faecalis v583	NA	EOT87933
	Not tested	NA	Tyrosine decarboxylase	Enterococcus faecium W54	NA	MH358384, MH358385
-	ECD_03365	ACT45166	Glutamate decarboxylase	Escherichia coli BW25113	100.00%	AIN33843
-	LVIS_1847	ABJ64910	Glutamate decarboxylase	Lactobacillus brevis W63	NA	
++	LVIS_2213	ABJ65263	Tyrosine decarboxylase	Lactobacillus brevis W63	100.00%	MH3583846
-	LVIS_0079	ABJ63253	Glutamate decarboxylase	Lactobacillus brevis W63	NA	
+++	PROSTU_02509	EDU59320	putative tyrosine decarboxylase	Providencia rettgeri DSM1131	83%	EFE55437
+++	RUMGNA_01526	EDN78222	Tryptophan decarboxylase	Ruminococcus gnauvs ATCC29149	100.00%	EDN78222

Supplementary Table 2. List of microbiota harboring other PLP-dependent amino acid decarboxylases tested in this study

* Protein identity from strain tested compared to cloned gene Williams et al.⁴. NA, not applicable.

	DDC	TDCEFS	TDCEFM	PTDCEFM
[E] (nM)	10	10	10	10
[S] (µM)	100	1000	1000	500
[Km] (µM)	87.3	3043.0	7244.0	448.1
HillSlope	1.16	1.6	1.3	1.1
IC50 (µM)	0.01	93.8	79.2	201.3
Ki=IC50/(1+([S]/Km))	0.005	70.6	69.6	95.1
[IC50]/[s]	0.01%	9%	8%	40%

Supplementary Table 3. IC50 curve parameters

The parameters were determined by fitting a sigmoidal-curve ([inhibitor] vs. normalized response) using Graphpad Prism. Reactions were performed in triplicate.

Supplementary Table 4. Sample information Parkinson's patients

ID	<i>tdc</i> -gene abundanc e	Levodopa/c arbidopa Tablets per Day	PD Onset (year) - First Diagnosed at Rush University	Disease Duration (years)	Date of Enrollment into Microbiota Research Study at Rush University	UPDRS*	Hy Stage**	Sex	Age	Race	BMI	Medication
Subject 1	1.55E-07	3 Tablets	2010	2	16-5-2012	35	2	Male	63	Caucasian	27.11	No; None
Subject 2	4.30E-07	11 Tablets	2001	11	25-10-2012	30	2.5	Male	50	Caucasian	24.28	No; None
Subject 3	6.48E-08	5 Tablets	2004	8	8-5-2012	36	2	Female	53	Caucasian	22.86	Yes; Naproxyn
Subject 4	8.97E-08	3 Tablets	2008	4	20-8-2012	13	2	Female	58	Caucasian	27.45	No; None
Subject 5	4.01E-07	4 Tablets	1998	14	20-9-2012	26	2	Female	68	Caucasian	23.92	Yes; Aspirin
Subject 6	2.37E-08	3 Tablets	2007	6	4-10-2012	35	2	Male	58	Caucasian	31.44	Yes; Aspirin
Subject 7	6.23E-07	10 Tablets	1991	22	14-6-2012	21	2	Female	57	Caucasian	18.01	No; None
Subject 8	8.69E-08	8 Tablets	2006	6	16-8-2012	27	2	Male	55	Caucasian	23.74	Yes; Aspirin
Subject 9	2.00E-07	6 Tablets	1997	15	18-9-2012	19	2	Male	82	Caucasian	29.12	No; None
Subject 10	2.75E-07	7 Tablets	2002	10	6-12-2012	40	2	Male	74	Asian	28.2	No; None

* Unified Parkinson Disease Rating Scale, ** Hoehn and Yahr Stage, BMI = Body Mass Index. According to medical records, all subjects were on the same levodopa/carbidopa dose thereafter their PD Onset (year) at Rush University Medical Center Neurology Department. Parkinson's disease was diagnosed according to the UK Brain Bank Criteria⁵.

Supplementary Table 5. Bivariate correlations

	Correlations										
		L-Dopa Tablets per Day	Disease Duration (years)	UPDRS	Hy Stage	Sex	Age	Race	BMI	Medica tion	
<i>tdc-</i> gene	Pearson Correlation	0.662*	0.823**	-0.195	0.351	-0.264	0.024	-0.071	-0.652*	-0.401	
abun dance	Sig. (2- tailed)	0.037	0.003	0.589	0.320	0.461	0.949	0.845	0.041	0.251	
	Ν	10	10	10	10	10	10	10	10	10	

* Correlation is significant at the 0.05 level (2-tailed). ** Correlation is significant at the 0.01 level (2-tailed).

Supplementary Table 6. Bacterial strains and Plasmids used in this study

Bacterial strains	Genotype	Reference
Escherichia coli DH5a	F ⁻ ; endA1; glnV44; thi-1; recA; relA1; gyrA96; deoR; nupG; purB20; φ 80dl acZΔM15; Δ (lacZYA-argF)U169; hsdB17(re ⁻ me ⁺): λ^-	6
Escherichia coli BL21 (DE3)	<i>E. coli</i> str. B; F ⁻ ; <i>ompT</i> ; gal; dcm; lon; hsdS _B ($r_B - m_B -$); λ (DE3 [lacI lacUV5-T7p07 ind1 sam7 nin5]); [malB ⁺] _{K-12} (λ^{S})	7
Escherichia coli BW25113	$lacI^+$; rrnB _{T14} ; $\Delta lacZ_{WJ16}$; hsdR514 $\Delta araBAD_{AH33}$; $\Delta rhaBAD_{LD78}$; rph-1; $\Delta (araB-D)567$; $\Delta (rhaD-B)568$; $\Delta lacZ4787$ (::rrnB-3)	8
<i>E. faecalis</i> v583/ATCC 700802		9
<i>E. faecalis</i> Δ TDC	$tyrS:nhaC(\Delta tdcA)$	10
Enterococcus faecium W54		Winclove Probiotic B.V.
Lactobacillus brevis W63		Winclove Probiotic B.V.
Ruminococcus gnavus ATCC29149		ATCC
Providencia rettgeri DSM 1131		DSMZ
<i>Bacteroides fragilis</i> ATCC 25285		ATCC
Plasmids used	Gene-insert	
pET15b		Novagen
pSK11	pET15b- ^P TDC _{EFM}	This study
pSK18	pET15b-TDC _{EFS}	This study
pSK22	pET15b-TDC _{EFM}	This study
pET15b- OHu25359C	pET15b-DDC cDNA	This study / GenScript

Component	g/L
Glucose	2.000
NaCl	0.080
K ₂ HPO ₄	5.310
KH_2PO_4	2.650
NaHCO ₃	0.400
Beef extract	5.000
Yeast extract	3.000
Peptone	0.600
CaCl ₂	0.008
MgSO ₄	0.008
Cysteine	0.500
Hemin	0.005
Vitamin solution (1000x)	
D-biotin	0.0020
D-Pantothenic acid	0.0100
Ca2.Nicotinamide	0.0050
Vitamin B12	0.0005
Thiamin.HCl	0.0040
Para-aminobenzoic acid	0.0050
Riboflavin	0.0050
Folic acid	0.0020
Pyridoxyal-5-Phosphate	0.0100
Vitamin K1	0.0005
Trace Elements (1000x)	
EDTA	1.000
$ZnSO_4.7H_2O$	0.178
MnSO ₄ .H ₂ O	0.452
FeSO ₄ .7H ₂ O	0.100
CoSO ₄ .7H ₂ O	0.181
CuSO ₄ .5H ₂ O	0.010
H ₃ BO ₃	0.010
Na ₂ MoO ₄ .2H ₂ O	0.010
NiSO ₄ .6H ₂ O	0.111

Supplementary Table 7. Constituents of enriched beef broth (EBB) medium used in this study

The KPO₄ solution is buffered at 50 mM pH7.

Supplementary Table 8. Primer sequences used in this study

Primers	5'-Sequence-'3	Target
sk075	AGGAGG <u>CTCGAG</u> AAAGATATGGATATCAAGGCCG	<i>E. feacium</i> W54 ^P TDC _{EFM}
sk076	AGGAGG <u>GGATCC</u> CCAGTATCACCGAAACATCC	<i>E. feacium</i> W54 ^P TDC _{EFM}
sk153	AGGAGG <u>CTCGAG</u> AAAAACGAAAAATTAGCAAAAGGCG	<i>E. faecalis</i> v583 TDC _{EFS}
sk154	AGCAGA <u>GGATCC</u> CAATCAGACGAACGTTCCCTC	<i>E. faecalis</i> v583 TDC _{EFS}
sk171	AGAGAG <u>CTCGAG</u> AGTGAATCATTGTCGAAAG	<i>E. feacium</i> W54 TDC _{EFM}
sk172	ATATAT <u>GGATCC</u> CCAAACATGCGTCAGAAACAG	<i>E. feacium</i> W54 TDC _{EFM}
qPCR	5'-Sequence-'3	Reference
DEC5f	CGTTGTTGGTGTTGTTGGCACNACNGARGARG	2
DEC3r	CCGCCAGCAGAATATGGAAYRTANCCCAT	2
Eub338	ACTCCTACGGGAGGCAGCAG	11
Eub518	ATTACCGCGGCTGCTGG	11

Underlined sequences represent restriction sites used.

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R Enterococcus sp. HMSC055603 + Enterococcus sp. HMSC055603 60b00 62b00 64b00 66b00		74000 76000	78000 8000	D 82000 840	00 86000	88000 900	DO 92000 940	00
Enterococcus sp. HMSC067C01 56000 58000 60000 62000		70000 72		6600 78600	80b00 82b00	84boo	seboo 88boo	90000
Enterococcus sp. HMSC076E04 + Enterococcus sp. HMSC065H12 00 4000 6000 8000 1000		18000			28000 300	00 32000	34000 36000	38000
A DOLOGIE SP. HMSC065H12 2000 4000 6000 8000 Control Con				100 24600 24			600 34600 34 	6000 1111 1111
2000 4000 6000 8000 R Enterococcus sp. HMSC065H03 + Enterococcus sp. HMSC065H03 → 38000 38000 40000 43000	44000 48000 48000	180 180 190 190 190 190 190	00 <u>54000</u> <i>e</i> °	000 58000 P	σός			
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Enterococcus sp. HMSC063C12 Enterococcus sp. HMSC076D08 jbo 40b0 60b0 80b0 100		, eddu 	22000	28000	28000 300	32000	34000 36000	38000
Enterococcus sp. HMSC076D08 -10000 -8000 -8000 -4000 -2		00 6000				1900 20000	22000 24000	26b0
Enterococcus sp. HMSC035C10 Enterococcus sp. HMSC035C10 2000 4000 6000 8000 R		18000 1	8000 20000 					
Enterococcus sp. HMSC070F12 + Enterococcus sp. HMSC070F12 -14000 -12000 -10000 -8000	-6doo -4doo -2doo	0 20					6000 18000 2 I	20000
				boo 24boo 2		30000 3	2000 34000 3	36000
		28000 280		500 34500 3		40000 4	2000 44000 4	46000
				00 240				

Enterococcus sp. HMSC074F07