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1 **Competitive selection of lactic acid bacteria that persist in the human oral cavity**

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3 Running title: oral persistence of lactic acid bacteria

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SUMMARY

Lactic acid bacteria (LAB) might offer opportunities as oral probiotics provided candidate strains would persist in the mouth. After intake of a mixture of 69 LAB, especially strains of *Lactobacillus fermentum* and *L. salivarius* were recovered. Co-aggregation with other microbes is likely not a prerequisite for persistence since *L. salivarius* strongly co-aggregated with typical oral cavity isolates whereas *L. fermentum* failed to display this phenotype.

Certain strains of lactic acid bacteria (LAB) are of interest as probiotics, which are defined as "live microorganisms that when administered in adequate amounts confer a health benefit on the host" (7). For oral health applications, despite broad interest of the scientific and industrial communities (2, 4, 17), functional criteria for selection of probiotics are in their infancy, and correlations between *in vitro* data and human intervention studies are scarce (6, 15). One potential mechanism of an oral probiotic is the inhibition of growth and maintenance of detrimental resident bacteria in specific oral sites. The screening of lactic acid bacterial species from oral cavities led to the identification of strains of *L. paracasei* ssp. *paracasei* and *L. rhamnosus,* which inhibited the growth of oral pathogens *in vitro*, including *Streptococcus. mutans* and *Porphyromonas. gingivalis* (20). Probiotic effects have also been demonstrated *in vivo*. The probiotic *S. salivarius* K12 is proposed to persist in the oral cavity where it changes the bacterial community and improves oral malodour parameters (1). Similar observations have been reported for *Weissella cibaria* (11).

For strategies to decrease the activity or abundance of the detrimental bacteria, colonization, or at least temporal persistence of probiotic bacteria is a phenotypic trait, which is highly likely to be required to achieve a functional health benefit (9, 19). The work presented here evaluates the competitive persistence of a range of LAB in the human mouth. A total of 69 food-grade, lactic acid bacteria (LAB) strains from the *Lactobacillus, Lactococcus* and *Streptococcus* genera were evaluated for their persistence *in vivo* in the human oral cavity. The strains were obtained from the NIZO culture collection as well as public culture collections (**Table 1**). Spontaneous rifampicin-resistant mutants were selected upon sub-culturing the wild-type strains in medium containing 10 µg/ml rifampicin and subsequently in 50 µg/ml rifampicin. The growth rates of the rifampicin resistant mutants were similar to wild-type cells in laboratory culture media (data not shown).

The rifampicin-resistant LAB strains were separately cultured overnight in the presence of 50 µg/ml rifampicin, washed, and mixed in a final volume of 30 ml of saline at a concentration of approximately $2x10^8$ cfu per strain. Ethical approval for human studies was given by the Commissie Mensgebonden Onderzoek regio Wageningen. Three subjects that were

previously confirmed to lack rifampicin resistant oral bacteria, held the mixture in their mouth for 1 minute, gently washing the liquid around their oral cavity, after which the mixture of bacteria was spit out. Saliva, tongue scrapings, and tooth swaps were collected by the subject after 5 min, 15 min, 1 h, 4 h, 24 h, 13 d and 28 d after administration. Tongue scrapers (DA retail B.V., Zwolle, The Netherlands) were rinsed in 5 ml saline, and swabs in 1 ml saline. The subjects did not consume any food, but were allowed to drink water, during the first 4 h after receiving the oral rinse, and subsequently no dietary or behavioral restrictions were imposed.

Enumeration of total rifampicin-resistant bacteria was performed on standard media containing 50 µg/ml rifampicin (**Fig 1**). The highest numbers of colonies from all three volunteers were recovered from saliva, ranging from 10^7 cfu/ml 5 min after rinsing to 10^5-10^6 cfu / ml 4 h later. In saliva samples, the numbers of rifampicin-resistant bacteria from subject 2 declined >10^5-fold within the first 24 hours whereas the colony-recovery in saliva samples from subjects 1 and 3 only dropped 10^3 fold. Dental swabs consistently contained lower amounts of LAB inoculants, and tongue scrapings showed considerable variation among the subjects. Rifampicin resistant bacteria were still recovered at 13 days after administration in the saliva from subjects 1 and 3 in concentrations of 5 x 10^1 and 7 x 10^3 cfu/ml saliva, and in subject 3 even after 28 days, indicating that in some individuals one or more of the administered strains display a very high level of persistence.

From each subject, thirty rifampicin-resistant bacterial isolates were selected on the basis of colony morphology, type of sample and time point (mostly 24 h after administration) of the LAB strains. Six isolates were collected at the 13 and 28 d time points. Species identification was performed using V1-V3 16S rRNA gene sequencing (12) (**Supplemental Table 1**). Fifty eight percent of the isolates were identified as being *Lactobacillus fermentum* while only 12% of the strains in the oral rinse were *L. fermentum.* Also strains of *L. salivarius*, and *L. (para)casei* were recovered frequently among the isolates. This result is in agreement with other studies reporting that these species are commonly found in the normal oral microbiota (14). Isolates of *L. brevis*, *L. delbrueckii*, *Lactococcus lactis* and *S. thermophilus* were not

among the 96 isolates examined, suggesting that they are unable to form persistent populations in the mouth.

Two discriminative colony-types of *L. fermentum* were isolated. GTG-5 PCR-identification (16) showed that these represented *L. fermentum* NIZO1220 (flat rough-edged colonies) and NIZO2930 (pink, large colonies) (**Figure 2**). For *L. salivarius*, molecular typing according to GTG-5 PCR was not sufficient. RAPD4 (5'- AAGAGCCCGT-3'), M13 (5'-GAGGGTGGCGGTTCT-3') and Box-A1R (5'- CTACGGCAAGGCGACGCTGACG-3') PCRs assisted in the partial differentiation of the *L. salivarius* strains recovered from the subjects (**Figure 3**). Six out of the nine *L. salivarius* oral isolates examined showed RAPD4 PCR patterns shared among *L. salivarius* strains NIZO880, NIZO881, and NIZO2938. The remaining *L. salivarius* isolates were likely strains NIZO2520 and/or NIZO2943. The diversity of *L. salivarius* in the recovered bacterial isolates suggest that *L. salivarius* strains commonly persist for extended periods in the oral cavity compared to the other species tested.

In a second human study, rifampicin resistant *L. fermentum* NIZO1220 and *L. salivarius* NIZO2521 were administered in concentrations of 10^9 cfu to the oral cavity of 5 subjects and the persistence of these strains was followed over time similar as described above. Surprisingly, rifampicin resistant colonies were recovered from the oral cavity of subject 1 prior to receiving the oral rinse. This subject was the same individual as subject number 3 in the initial oral persistence trial. Identification by 16S rRNA gene sequencing and RAPD-PCR methodology showed that this individual harbored at least two different strains of rifampicin-resistant *L. salivarius* which were distinct from strain NIZO2521 (data not shown). A similar long persistence was reported for *Lactobacillus rhamnosus* GG that was identified in saliva from a female subject 5 months after he use of LGG (21).

For at least 24 h after administration, the inoculated strains were found in amounts of 10^2-10^5 cfu/ml saliva (**Fig. 4**). Thereafter, *L. fermentum* or *L. salivarius* strains were ranging from between 10 and 1000 cfu/ml saliva at 2 and 5 days after administration, and returned to

base-line levels in each of the subjects within 15 days although a high inter-individual variation was observed.

L. fermentum NIZO1220 and *L. salivarius* NIZO2521 were individually counted in samples on basis of colony morphology. Since subject 1 had rifampicin-resistant bacteria in the mouth prior to taking the oral rinse, this subject was excluded from further analysis at the group level. In the majority of samples, *L. fermentum* NIZO1220 was recovered in 1 to 2 log higher numbers as compared to *L. salivarius* NIZO2521, although not always significant (**Figure 5).** These findings confirm that *L. fermentum* NIZO1220 and *L. salivarius* NIZO2521 are LAB with relatively high persistence capacities in the human oral cavity.

Previous studies evaluating individual strains have shown variable capacities of LAB to colonize the human mouth. *L. reuteri* ATCC 55730 that was associated with an *in vivo* reduction of *S. mutans* (18) disappeared in almost 50% of subjects within 24 h (3). LGG was maintained in only 66% of the participating subjects after the first day of discontinuation of its intake (21). Our study is in line with these observations, since the same strains of the species *L. rhamnosus* and *L. reuteri* were included in our initial collection of strains. In contrast, *S. salivarius* K12 persisted in the human oral cavity for a period of up to two weeks (1).

Co-aggregation is proposed as a mechanism by which oral bacteria adhere to each other and as a result may colonize persistently in biofilms in the host oral cavity (13). For example, the capacity of orally administered *Weissella cibaria* isolates to inhibit resident oral bacteria is proposed to be at least partially determined by the capacity of these bacteria to co-aggregate with target strains including *F. nucleatum*, *T. denticola*, and *P. loescheii* (11, 13). To evaluate whether adherence to other oral bacteria might be a factor influencing the persistence characteristics of LAB in the mouth, the ability to adhere and co-aggregate with oral bacteria was investigated for the 2 most persistent strains of *L. fermentum* and all 6 *L. salivarius* strains included in the oral rinse. Co-aggregation capacity of lactic acid bacteria

was performed with cultured representatives of common oral microorganisms that are implicated as causative agents of bad breath or caries **(Table 2)**.

L. salivarius NIZO2520, NIZO2521, and NIZO2943 co-aggregated with the majority of the target strains, with the exception of *S. mutans* and *P. melaninogenica* (**Table 3**). Small aggregates indicated that *L. salivarius* NIZO2521 also co-aggregated slightly but significantly with *P. melaninogenica* HG73 (**Supplemental Figure 1**). In comparison, *L. fermentum* strains NIZO1220 and NIZO2930 and *L. salivarius* strains NIZO880, NIZO881 and NIZO2938 did not co-aggregate with any of the oral strains. Possible explanations for the persistence of *L. fermentum* may be the ability to adhere to species that were not tested, or directly to dental surfaces, e.g. by adhesion to salivary proteins. Indeed, *in vitro* assays revealed a considerable degree of variation of adherence of individual bacterial strains to salivary proteins (10), which indicates that co-aggregation is not the sole mechanism by which bacteria can persist in the oral cavity.

One important caveat which might prevent the use of *Lactobacillus* as oral probiotics is that members of this genus have also been associated with childhood caries because of their strong acidifying characteristics, although their presence was not sufficient to explain all cases of caries (8). Therefore, probiotic characteristics of the selected strains should be carefully monitored *in vivo*, e.g. for the absence of a contribution to dental decay, and not only based on *in vitro* characteristics.

In conclusion, the ability of a bacterial strain to persist in the oral cavity is likely to support oral-probiotic efficacy. The approach we presented here can serve as an initial step in the selection of candidate probiotic strains aiming to promote oral health. *L. fermentum* and *L. salivarius* strains display the best extended oral persistence relative to other LAB. Further evaluation of these strains should examine their effects on the composition and activity of the endogenous oral microbiota and should be complemented with determination of the possible consequences for certain health parameters including exhaled VSC, reduced levels of *S. mutans*, or other clinically relevant characteristics.

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Table 1. LAB examined for persistence in the human mouth. The LAB strains were routinely grown in preferred laboratory culture media under anaerobic conditions (90% N_2, 5% H_2, and 5% CO_2). Streptococci and lactococci were grown in M17 medium (Oxoid, Hampshire, UK) supplemented with 1 % lactose (or glucose when mentioned) at 30 °C and 42 °C, respectively. Lactobacilli were grown in MRS medium (Merck, Darmstadt, Germany). at 37 °C.

Species	**source**	**Alternate**	**origin**
L. acidophilus	NIZO867	LMG 7943, DSM 20079	N/A = not known
L. acidophilus	NIZO221	ATCC4357	N/A
L. acidophilus	NIZO222		N/A
L. acidophilus	NIZO223		N/A
L. acidophilus	NIZO225		N/A
L. acidophilus	NIZO229		N/A
L. acidophilus	NIZO267		N/A
L. brevis	NIZO2927	NCIMB 8840	human saliva
L. brevis	NIZO289		cheese
L. brevis	NIZO2019		cheese
L. brevis	NIZO1322	LMG 7944, DSM 20054	human feces
L. brevis	NIZO293		cheese
L. brevis	NIZO2491		pork pickeled sausage
L. bulgaricus	5.2		Campina starter culture
L. bulgaricus	2.3		Campina starter culture
L. casei ssp. *Casei*	NIZO2928	NCIMB 8822	human saliva
L. casei ssp. *Casei*	NIZO2929	NCIMB 8823	human saliva
L. casei ssp. *Casei*	NIZO637		N/A
L. casei ssp. *Casei*	NIZO889		N/A
L. casei ssp. *Casei*	NIZO931		N/A
L. delbrueckii ssp. *lactis*	NIZO235	ATCC7830	N/A
L. delbrueckii ssp. *lactis*	NIZO2944	DSM 20073	saliva
L. fermentum	NIZO2930	NCIMB 701751	saliva
L. fermentum	NIZO2931	NCIMB 700335	human oral strain
L. fermentum	NIZO2517	LMG 9846	saliva
L. fermentum	NIZO2932	NCIMB 8828	human saliva
L. fermentum	NIZO2933	NCIMB 8829	human saliva
L. fermentum	NIZO2934	NCIMB 8830	human saliva
L. fermentum	NIZO307	ATCC9338	human oral cavity
L. fermentum	NIZO1220	LMG11441	N/A
L. paracasei ssp. *paracasei*	NIZO2935	NCIMB 700680	oral source
L. paracasei ssp. *paracasei*	NIZO2936	NCIMB 702713	Child saliva
L. paracasei ssp. *paracasei*	NIZO2518	DSM 20020	Child saliva
L. paracasei ssp. *paracasei*	NIZO2945	DSM 4905	oral cavity
L. paracasei ssp. *paracasei*	NIZO1480	DSM 20244	Milk
L. paracasei ssp. *paracasei*	NIZO632		N/A
L. paracasei ssp. *paracasei*	NIZO1353	DSM 5622, ATCC25302	N/A
L. pentosus	NIZO2514		bamboo shoot pickled
L. plantarum	NIZO631		N/A

L. plantarum	NIZO2519	LMG 9212	human saliva
L. plantarum	NIZO1315		N/A
L. plantarum	NIZO1699		soakwater of soy beans
L. plantarum	NIZO1317	DSM 20174, LMG6907	pickeled cabbage
L. plantarum	NIZO2029		Raw-milk cheese
L. plantarum	NIZO1843		N/A
L. plantarum	NIZO2484		pork pickled sour sausage
L. plantarum	NIZO2260	299v, DSM 9843	human intestine
L. plantarum	NIZO2500		pork pickled sour sausage
L. plantarum	NIZO2532		shrimp pickled sausage
L. plantarum	NIZO1836	NCIMB 8826, WCFS1, LMG9211	human saliva
*L. reuteri**	NIZO2691		(biogaia product) breast milk
*L. rhamnosus**	NIZO1665	LGG	human origin
L. salivarius	NIZO880		human intestine
L. salivarius	NIZO881		human intestine
L. salivarius ssp. *salivarius*	NIZO2938	NCIMB 8816	human saliva
L. salivarius ssp. *salivarius*	NIZO2521	DSM 20555	Saliva
L. salivarius ssp. *salivarius*	NIZO2520	DSM 20554	Saliva
L. salivarius ssp. *salivarius*	NIZO2943	DSM 20492	human saliva
Lactococcus. lactis ssp. *Cremoris*	NIZO42		N/A
Lactococcus lactis ssp. *Cremoris*	NIZO47		Starter
Lactococcus lactis ssp. *Cremoris*	NIZO57		N/A
Lactococcus lactis ssp. *Cremoris*	NIZO706		N/A
Lactococcus lactis ssp. *Diacetylactis*	NIZO86		starter
Lactococcus lactis ssp. *Lactis*	NIZO2051		raw-milk curd
Lactococcus lactis ssp. *Lactis*	NIZO8	R5	N/A
Lactococcus lactis ssp. *Lactis*	NIZO14		N/A
S. thermophilus	NIZO133		N/A
S. thermophilus	NIZO2269		N/A
S. thermophilus	NIZO122		raw-milk cheese

*Included for reference purposes

Table 2 Strains of oral bacteria used in this study. *Streptoccocus mutans* was grown on M17 containing 1% glucose at 37 °C. The other strains (Table 2) were grown in BHI medium (Merck, Darmstadt, Germany) at 37 °C.

Species	**Strain ID**	**Source**
Porphyromonas gingivalis	HG66	ACTA, Amsterdam
Porphyromonas endodontalis	HG181	ACTA, Amsterdam
Prevotella intermedia	HG110	ACTA, Amsterdam
Prevotella melaninogenica	HG73	ACTA, Amsterdam
Peptostreptococcus anaerobius	HG578	ACTA, Amsterdam
Fusobacterium nucleatum	HG646	ACTA, Amsterdam
Tannerella forsythia	HG1245	ACTA, Amsterdam
Streptococcus mutans	UA 159	ACTA, Amsterdam
Streptococcus mutans	NIZO B1215	NIZO culture collection
Streptococcus mutans	C180-2	ACTA, Amsterdam

Table 3. Co-aggregation of mixtures of *Lactobacillus* and oral bacteria.

	L.salivarius NIZO2521	*L.salivarius* NIZO2520	*L.salivarius* NIZO2943
Control[a]	0[b,c]	0	0
F. nucleatum HG646	3	3	3
P. anaerobius HG578	3	3	3
P. endodontalis HG181	2	2	2
P. gingivalis HG66	4	4	4
P. intermedia HG110	3	3	3
P. melaninogenica HG73	0	0	0
S. mutans B1215	0	0	0
T. forsythia HG1245	4	3	3

[a] *L. salivarius* without oral bacteria

[b] Data are provided for co-aggregation after 2 h of incubation. These results are consistent with the findings observed at 4 h and 24 h (data not shown).

[c] Scores are based on visual inspection, using the following scoring criteria (5): 0 = no visible aggregates in the cell suspension, 1 = small uniform co-aggregates in suspension, 2 = definite co-aggregates easily seen but suspension remained turbid, 3 = large co-aggregates which settled rapidly leaving some turbidity in the supernatant fluid, 4 = clear supernatant fluid and large co-aggregates which settled immediately.

224

Figure legends

Figure 1 Total numbers of rifampicin resistant LAB recovered from the oral cavity at different times during the first 24 hours after administration. The saliva (A), tongue (B) and teeth (C) of the three subjects (subject 1, diamonds; subject 2, squares; subject 3, triangles) enumerated independently. Limit of detection was 10 cfu per ml of sample.

Figure 2 Dendrogram and GTG5 PCR fingerprints for comparison of *L. fermentum* strains included in the oral rinse and isolates from the oral cavity collected during the 1st persistence trial. For strains, NIZO numbers are denoted. Isolates are indicated by subject number and isolate number.

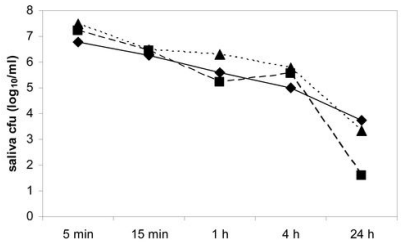
Figure 3. Dendrogram and PCR fingerprints for comparison of *L. salivarius* strains included in the oral rinse and isolates from the oral cavity collected during the 1st persistence trial. The comparison is based on the combined PCR fingerprints obtained by RAPD4, M13, and BOX-A1R.

Figure 4 Recovered total numbers of rifampicin resistant colonies at different time points in five subjects (subject 1, closed diamonds; subject 2, closed squares; subject 3, closed triangles; subject 4, open circles; subject 5, asterix) and two sampling sites were enumerated independently (A: saliva, and B: tongue scrapings). Limit of detection was 10 cfu per ml of sample.

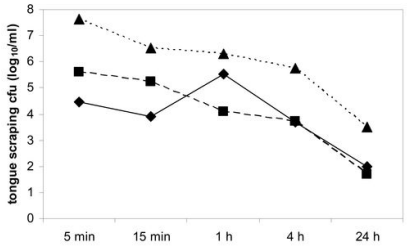
Figure 5 Relative persistence (cfu/ml) of *L. fermentum* NIZO1220 (black bars) and *L. salivarius* NIZO2521 (dashed bars) in the oral cavity of 4 healthy human subjects (subjects 2-5), as measured in saliva (A) and tongue scrapings (B). No rifampicin bacteria were recovered from subject 2 to 5 before oral administration of the two candidate probiotic strains. Subject 1 harbored rifampicin-resistant bacteria before administration, and was therefore excluded from the analysis. Limit of detection was 10 cfu per ml of sample.

Snel et al. Figure 1

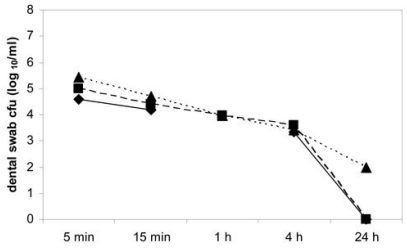
A



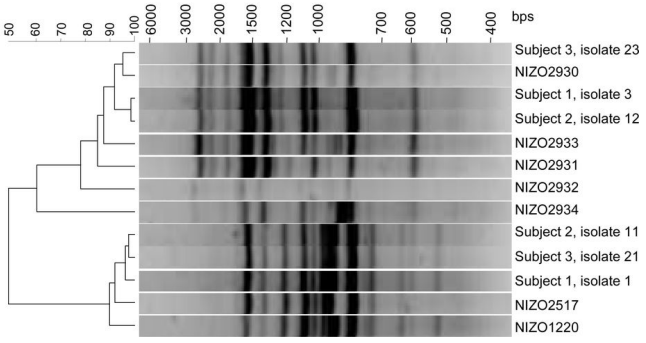
B



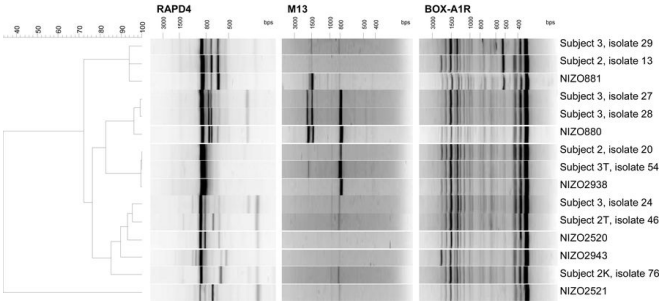
C



Snel et al. Figure 2

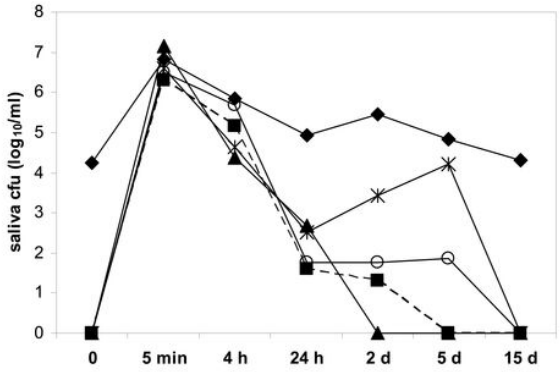


Snel et al. Figure 3

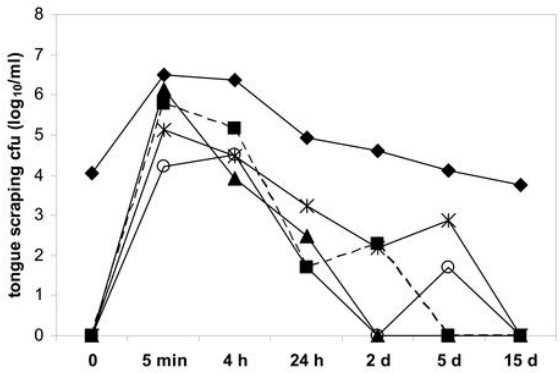


Snel et al. Figure 4

A

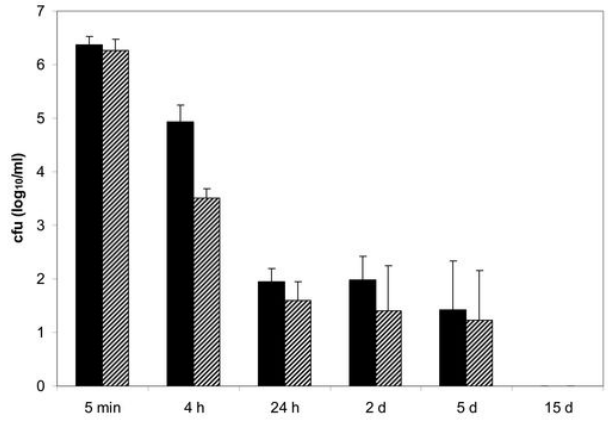


B



Snel et al. Figure 5

A



B