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# SYMBIOTIC BACTERIA AND THEIR THERAPEUTIC POTENTIAL

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## INTRODUCTION

The majority of probiotic products currently on the market for human use are based on Lactic Acid bacteria (Lactobacillales), and many contain species of the genera *Lactobacillus* or *Lactococcus* (members of the phylum of Firmicutes) or *Bifidobacteria* (which were reclassified from '*Lactobacillus bifidum*' to a genus within the Actinobacteria phylum). What these bacteria have in common is their production of lactic acid as a result of carbohydrate fermentation. They are also frequently used as starter cultures for dairy products and other fermented foods.

Other, less frequently encountered probiotic bacterial species are apathogenic strains of *Enterococcus faecalis* (another Lactobacillales) and *Escherichia coli* (a Gamma-proteobacterium). On the market are products containing live *Enterococcus faecalis*, live *Escherichia coli*, and a bacterial lysate of *Enterococcus faecalis* and *Escherichia coli* (see chapter: "Identification of bacterial products" described later in this publication).

## *ENTEROCOCCUS FAECALIS* PREPARATION

### Animal studies with *Enterococcus faecalis* SY

Originally, the product based on apathogenic *E. faecalis* was described as 'oral vaccine' against enteropathogens such as *Salmonella*. Results obtained with mouse experiments were summarized in a publication that demonstrated some protection against *S. typhimurium* challenge and protection against intraperitoneal challenge with *Haemophilus influenzae* was also shown (*Rusch* et al., 1983). The presumed protection by probiotic bacteria against infections had already been proposed by Alfred Nissle, who explained the observed effect by *in situ* competition (*Nissle*, 1916). Instead, Rusch and co-workers preferred the hypothesis that the effect was the result of stimulation of 'non-specific immune activities'. This hypothesis was investigated in a follow up murine study that used *E. faecalis* SY (*Hyde* et al., 1986). Mice received the *E. faecalis* bacteria (still called *Streptococcus faecalis* in those days) via their drinking water for 3 weeks, after which the animals were sacrificed to isolate polymorphonuclear leukocytes (PMNs), macrophages and Natural Killer (NK) cells. The PMNs of treated animals were able to kill *Staphylococcus aureus* bacteria more efficiently than PMNs from untreated mice. However, neither the intracellular killing of *S. typhimurium* in macro-

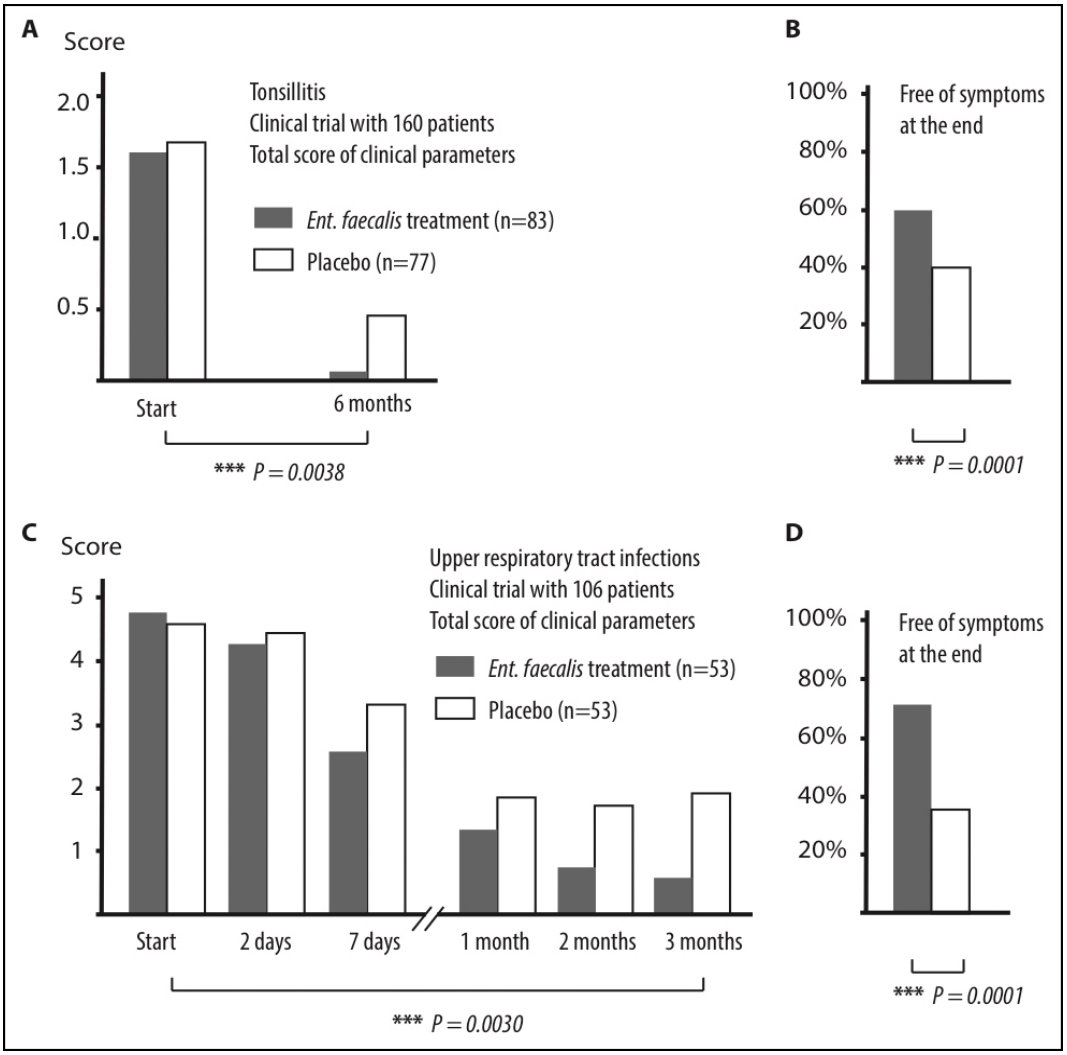


**Figure 1:** Results from two clinical trials with *Enterococcus faecalis* SY.
Panels A and B summarize findings for a trial with 160 patients suffering from chronic tonsillitis, Panels C and D results from the trial with 106 patients with frequent upper respiratory tract infections. Shown are total clinical scores (Panels A and C), and the percentage of patients free of symptoms at the end (Panels Band D). (Modified after *Rusch* et al., 1986; *Kalinski*, 1986, 1987).

phages, nor the activity of Natural Killer (NK) cells was enhanced as a result of the treatment (*Hyde* et al., 1986).

An animal study using 8-12 months-old pigs was used to investigate the immune modulation of *E. faecalis* SY in more detail. Animals were given various doses of the product to determine that twice a daily dose of $10^9$ bacteria per animal was required to produce increased cytotoxicity of peripheral granulocytes (*Ottendorfer* et al., 1995). Gut-associated lymphoid tissue (GALT) was isolated *post mortem* to assess local, short-term effects of presence of these probiotic bacteria. The PHA stimulation of lymphocytes isolated from the mesenteric lymph nodes was increased in exposed animals, while the nodes also contained more IgA-producing cells. Moreover, the s-IgA

levels in saliva were elevated (*Ottendorfer* et al., 1995). All these effects were considered to be in line with a mechanistic immune-modulation by *E. faecalis* SY that could explain its positive effects on human conditions such as COPD and recurrent airway infections.

**Clinical trials with *Enterococcus faecalis***

Two double blind placebo-controlled clinical trials were summarized in an early publication from 1986, one describing 160 patients with chronic tonsillitis and the other involving 106 patients suffering from various respiratory tract infections (*Rusch* et al., 1986). In both trials, the patients were treated with *E. faecalis* SY or placebo (the tonsillitis trial treatment lasted 6 months, in the other trial patients were treated for 3 months). In both trials, the immunological outcome was determined by the Merieux Multitest, a multi-antigen skin test that was frequently used in those days, and by assessment of the patient's health status by a physician. Further, immunoglobulin titres were determined in saliva and serum. At the end of the tonsillitis trial, which is described in more detail elsewhere (*Kalinksi*, 1986), both patient groups had improved their total scores for clinical parameters, but the group receiving treatment had significantly better scores ($p = 0.0038$) compared to the placebo group. Moreover, 65% of the patients receiving the probiotic were free of symptoms at the end of treatment, compared to 35% of the placebo group ($p=0.0001$). The key data from this trial are summarized in Figure 1 panels A and B.

The other trial targeted patients with 'impaired immune conditions' as judged by the frequency of chronic upper respiratory tract infections (*Kalinksi*, 1987). Again the difference in total clinical scores was highly significant between treatment and placebo group ($p=0.0003$), and in this trial the Merieux Multitest resulted in significantly higher reactivity in the treatment group ($p=0.0280$), while serum levels of IgA and IgM significantly increased compared to placebo. In the verum group 40 patients became free of symptoms (61%) compared to 20 patients in the placebo group (38%, $p=0.001$). This is summarized in Figure 1 panels C and D. From these two trials it was concluded that *E. faecalis* SY provided an effective and safe treatment for chronic upper respiratory tract infections due to immunodeficiencies (*Rusch* et al., 1986).

The level of circulating antibodies was subject of a study by Jansen and co-workers, who determined humoral IgG levels directed against *E. faecalis* in 10 healthy volunteers who daily took $10^7$ bacteria of *E. faecalis* SY for 3 weeks (*Jansen* et al., 1993). Their post-intake serum IgG levels were reduced compared to two samples taken (5 weeks apart) prior to intake, and the reduction was even stronger 3 weeks after the last intake, while IgG levels returned back to their original values 6 months later. The decrease in circulating IgG levels was interpreted as the result of an anti-inflammatory effect by the product (*Jansen* et al., 1993).

Another double-blind, placebo-controlled clinical trial was performed to evaluate the effect of *E. faecalis* SY on chronic recurrent bronchitis (*Habermann* et al., 2001). A total of 136 patients were randomly divided over a placebo group of 66 and a treatment group of 70 individuals. The treatment or placebo intake lasted for 6 months, after which the groups were followed for another 8 months. The treatment group suffered from 39 bronchitis incidents, which was 60% less than the 66 cases occurring in the placebo group

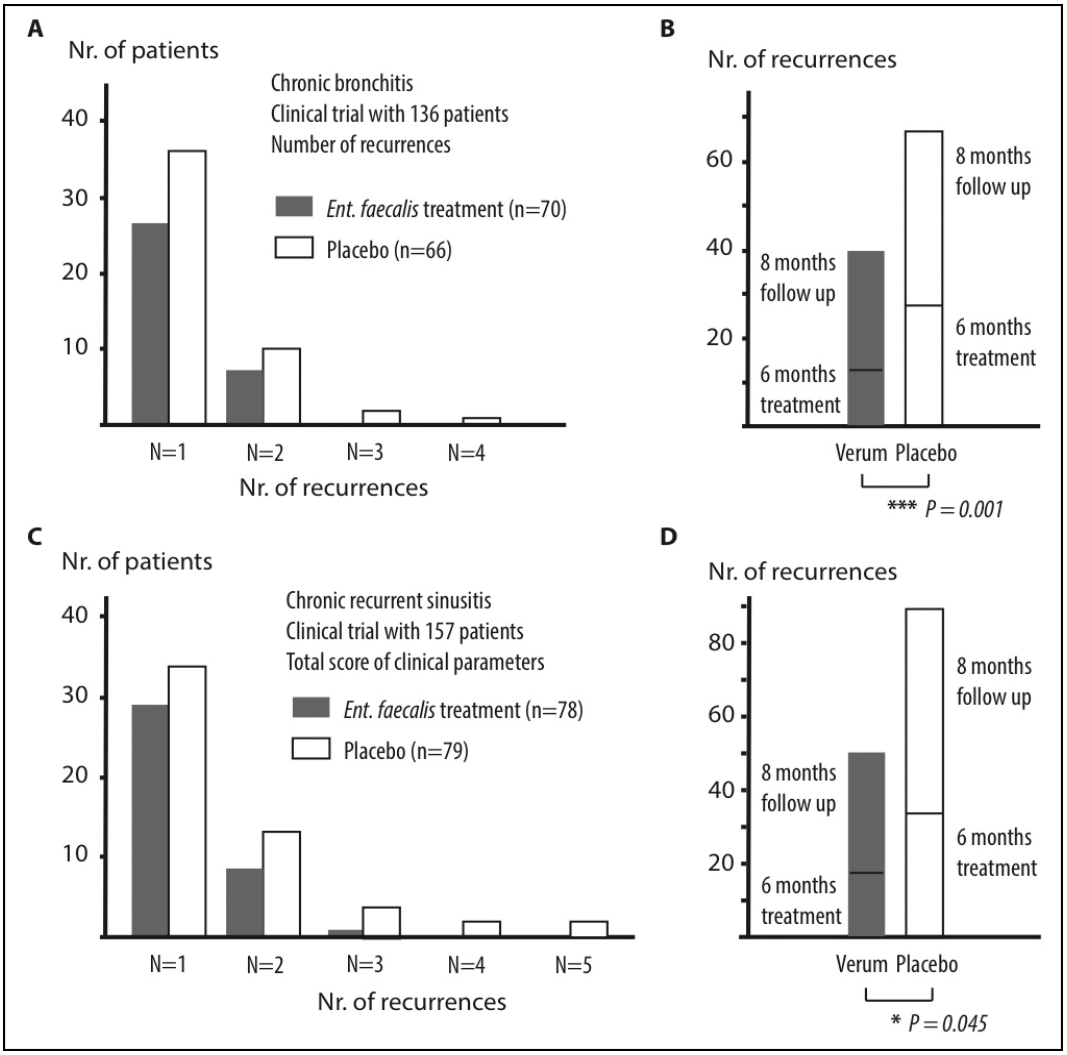


**Figure 2:** Results from two clinical trials with *Enterococcus faecalis* SY.
Panels A and B summarize findings for a trial with 136 patients suffering from chronic bronchitis. Panels C and D reports results from the trial with 157 patients with frequent sinusitis. Shown are the number of recurrences (Panels A and C), and the total number of recurrences during the 6 months of treatment and 8 months of follow-up (Panels B and D). The latter show P-values by Kaplan-Meier analysis. (Modified after *Habermann* et al., 2001, 2002).

(Figure 2, panels A and B). Treatment with the product further resulted in delayed relapse, fewer episodes, and less severe symptoms, compared to the placebo group. Side effects were not observed in either group (*Habermann* et al., 2001). Another publication from the same research group came out a year later, describing 157 patients suffering from chronic recurrent hypertrophic sinusitis (*Habermann* et al., 2002). The followed procedure was similar to the previous study, and this time 50 sinusitis incidents were observed in the treatment group of 78 patients, compared to 90 cases occurring in the placebo group of 79 patients, a significant difference (Figure 2 panels C and D). A difference in frequency of sinusitis was observed during treatment

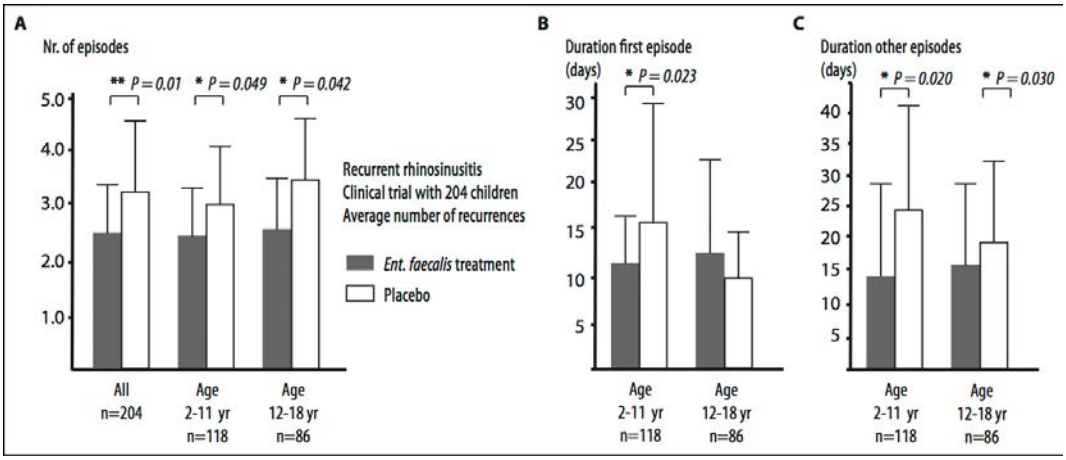


**Figure 3:** Results of a clinical trial with *Enterococcus faecalis* SY involving 204 children suffering from recurrent rhino-sinusitis.
Panel A reports the average number of episodes for all patients and for the two age groups that were separately analysed. Panel B shows the average duration of the first episode, and Panel C the average duration of other episodes, both for the two age groups. (Modified after *Kitz* et al., 2012).

as well as during the 8 months thereafter and, as with recurrent bronchitis, the time it took for the first relapse to occur was longer after treatment (*Habermann* et al., 2002).

Kitz and colleagues determined the efficacy of *E. faecalis* SY to treat recurrent sinusitis in children (*Kitz* et al., 2012). The study was a prospective trial, in which children suffering from recurrent rhino-sinusitis were assigned to standard treatment, with or without a subsequent 60-days course of the probiotic bacteria. Children in the treatment group had fewer episodes than the controls (p=0.01). The duration of the first episode was significantly shorter in the younger treatment group, and duration of other episodes was shorter for both age groups (Figure 3). A delay in onset of the first relapse and reduced severity of symptoms was marginally different between the two groups (*Kitz* et al., 2012).

A systematic review that addressed the application of probiotics for prevention of respiratory tract infections was published in 2009 (*Vouloumanou* et al., 2009). A total of 109 publications were identified in PubMed, of which 14 described randomised clinical trials (RCTs) that fulfilled all criteria for inclusion; this included the Habermann 2002 study. Most other included RCTs had tested *Lactobacillus* species, while two trials had used *Bifidobacterium* species. In contrast to the results reported by Habermann and colleagues, 10 of the 14 compared studies reported no difference in incidence of respiratory tract infections between treatment and placebo control group (*Vouloumanou* et al., 2009).

## Mechanistic model of action of *E. faecalis* SY

The mechanism of action of *E. faecalis* SY can be summarized as schematically presented in Figure 4. The bacteria activate monocytes/macrophages, which react by production of interleukin-1β (IL-1β) and IL-6. This stimulates the activation and subsequent proliferation of dormant B cells, with the production of IgA and s-IgA as a consequence.

This model is based on several independent observations. That *E. faecalis* SY bacteria resulted in IL-1β and IL-6 release by human PMNs was demon-

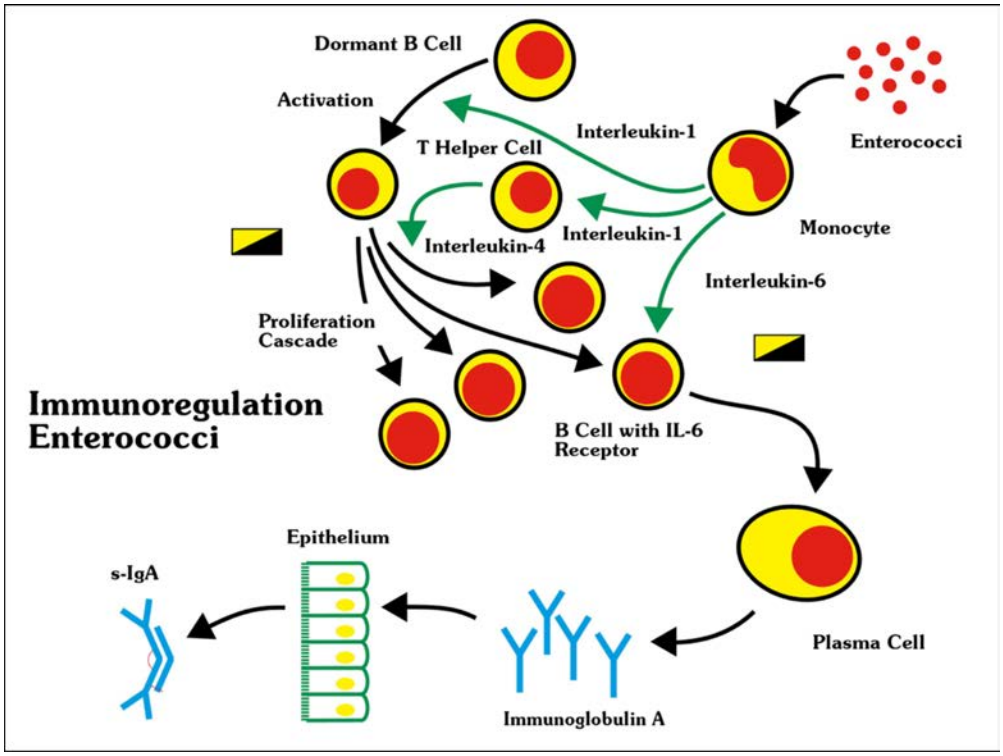


**Figure 4:** Model of immunoregulation by *Enterococcus faecalis* SY.
For explanation see text. (Taken from *Rusch* and *Zimmermann*, 1995).

strated *in vitro*; the cytokine production was dose-dependent and could be inhibited by dexamethasone but not by cyclosporine A (*Rosenkranz* and *Grundmann*, 1994). Production of interferon gamma (IFN-γ) was weaker but also dose-dependant, and could be inhibited by both dexamethasone and cyclosporine A. Proliferation of B cells was demonstrated in the pig model described above, while increased s-IgA levels in saliva were also demonstrated (*Ottendorfer* et al., 1995). The human clinical trials had produced data on an increase of serum IgA and IgM (*Rusch* et al., 1986; *Kalinksi*, 1986, 1987).

A random selection of the tonsillitis patients who had participated in the clinical trial previously mentioned (*Rusch* et al., 1986) had donated blood before the beginning of treatment, at 4 timepoints during treatment and again 3 months after treatment was finished. Monocytes isolated from these blood samples were tested for their production of IFN-γ, as well as IL-2, IL-1β and IL-6 (R. Kunze and H. Skarabis, unpublished data). As expected, the levels of these immune responses varied considerably between individuals, and some levels were too high or too low to be determined quantitatively by means of a standard curve. For those measures that were accurate, the trends prior to treatment and during treatment were compared for patients from the verum and treatment groups. Some of the obtained data are summarized in Figure 5. Notably, the production of IFN-γ in response to PHA+ *E. faecalis* SY stimulation was down-regulated in monocytes obtained from treated patients, compared to controls. This is shown in Panels 5A-C, illustrated by the decreased slope of the red curve (verum group) in panels B and C.

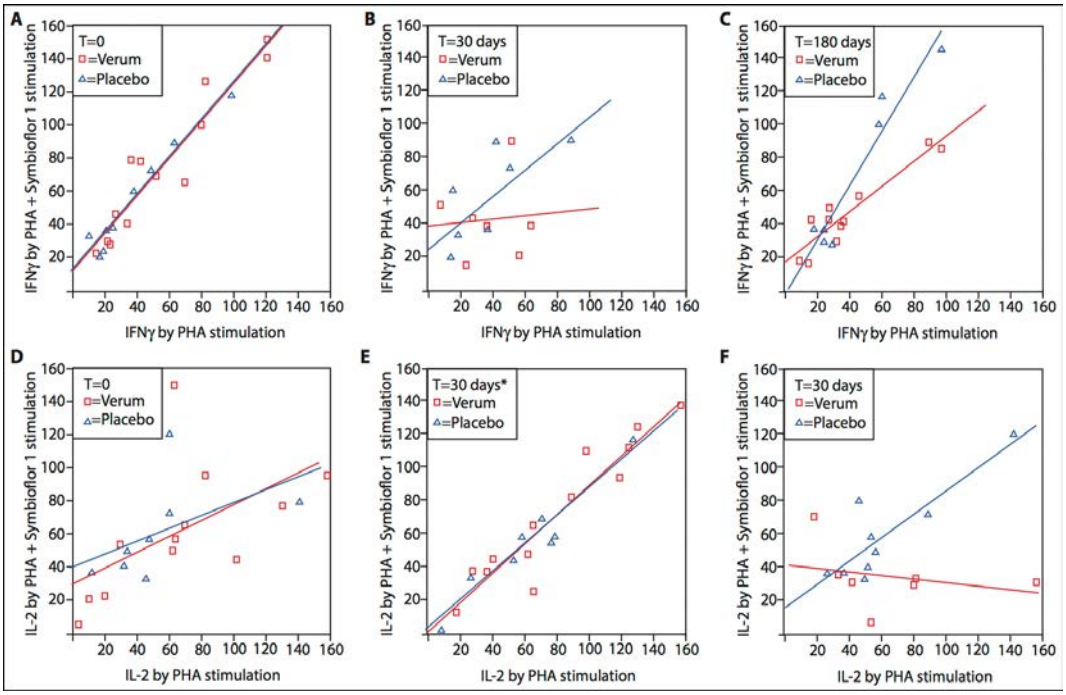


**Figure 5**: Production of immune regulators by monocytes isolated from patients who participated in the tonsillitis trial.
Panels A-C show the IFN-γ production after stimulation with PHA plotted against stimulation with PHA combined with *E. faecalis* SY. Panels D-F show the results for IL-2 production. Blood samples were taken prior to treatment (Panels A, D), after 30 days of treatment (Panels B, E, F) and after 180 days (panel C). Note that Panel E shows the outcome read after 15 hrs. of stimulation (marked by *), while D and F are read after 48 hrs. of stimulation. (Modified after R. Kunze and H. Skarabis, unpublished data).

When the levels of produced IL-2 were measured after 15 hrs. of incubation, they remained more or less the same over time, either in the verum or placebo group (data are shown for day 30 only, panel 5E, where the verum and treatment curve overlap), whereas the production levels of IL-2 differed significantly between the two treatment groups when read after 48 hr. This is shown in Panel 5F. Levels for IL-1β and IL-6 did not produce significant or consistent results (data not shown).

## Strain characterization and genome sequence

The product *E. faecalis* SY is a combination of 10 subcultures of *E. faecalis* strains, that are all highly similar (sometimes denoted S01 to S10). They were originally isolated from the stool of a healthy individual. Two of these have such a high resemblance to starter culture strains of *E. faecalis*, that it is assumed the individual had been colonised with a diary strain; the eight other subcultures all resemble each other and are only slightly different from the other two. The strains are all free of plasmids.

Two genome sequences have been obtained, representative of the clones that form the minority (*Domann* et al., 2007, *Fritzenwanker* et al., 2013), and a third sequencing project is underway that covers two other clones, including one of the majority type. Domann and colleagues presented their genome

sequence in 702 contigs, but the sequence has not been submitted to public databases. In their publication they compared the strain to clinical isolate *E. faecalis* V583 (a well-researched VanA-resistant blood isolate), whose genome was used as a template for assembly (*Domann* et al., 2007). Notably, two gaps were identified representing sequences present in V583 but absent in the *E. faecalis* SY genome. Since these two regions had an aberrant GC content, they were interpreted as genome islands obtained by V583 by horizontal DNA uptake. A number of virulence genes are found in these regions in V583 that are absent in *E. faecalis* SY, such as cytolysin (a toxin that disrupts membranes of other bacteria, erythrocytes and other eukaryotic cells), gelatinase (a secreted bacterial metallo-proteinase that catabolises gelatine, collagen, fibrinogen, casein and insulin, helping the bacteria to spread in the host), hyaluronidase (an enzyme allowing the use of hyaluronic acid as a carbon source), and enterococcal surface protein (assisting in adherence). Since the sequence of *E. faecalis* SY was not fully covered, absence of cytolysin and gelatinase was confirmed by PCR analysis (*Domann* et al., 2007). The *E. faecalis* SY genome does, however, contain the genes coding for aggregation substance (AS, coded by *agg*) and collagen adhesion factor (*ace*), both involved in binding to abiotic surfaces and to eukaryotic cells, and associated with virulence in pathogenic *E. faecalis* strains. These and other genes would support the ability to colonise the intestinal epithelium. Although it was described in the publication that the bacteria were capsulated (which had been postulated for some strains of *E. faecalis* SY [*Kropec* et al., 2005]), the genes responsible for capsulation were not found in the obtained genome sequence. Furthermore, it was determined that the sequenced strain was unable to form biofilms *in vitro* (*Domann* et al., 2007).

In 2013, the complete genome sequence of *E. faecalis* DSM 16431 was released, and although it was not mentioned in the genome announcement, this represents the same strain that Domann and colleagues had sequenced (*Fritzenwanker* et al., 2013). The complete circular genome (GenBank accession nr. HF558530.1) measures 2,810,675 bp, bearing 2733 recognized protein-coding sequences, 4 rRNA loci and 63 tRNAs. The sequence confirmed absence of the *vanB* operon and the pathogenicity island previously noticed by Domann and co-workers. Absence of capsule genes was also confirmed, as well as absence of gene *cas* (an essential gene for functional Cas-CRISPR activity). A unique integrated bacteriophage sequence of 45 kb was recognized that is absent in reference genome V583 (*Fritzenwanker* et al., 2013). *In silico* MLST confirmed the sequence type (ST) of the sequenced strain as ST248. Recently, the draft genome sequences of two other subcultures of *E. faecalis* SY were obtained; DSM16434 resembles DSM16431, while the DSM16430 represents one of the eight other subcultures that are all highly similar to each other. The notable difference between DSM16430 compared to the other two is that it lacks the gene for aggregation substance, and that the prophage is absent (*Fritzenwanker* et al., 2016).

**Safety and colonisation aspects of *Enterococcus faecalis* SY**

An *in vitro* opsonophagocytic test (the capacity of PMNs to take up and kill bacteria in presence of serum) was used to compare the serum resistance of *E. faecalis* SY bacteria with other *Enterococcus* strains, as a measure for safety of the product. This demonstrated that

*E. faecalis* SY is serum sensitive (*Kropec* et al., 2005).

Since virulent *E. faecalis* strains are frequently causing urinary tract infections (UTIs), in particular in nosocomial settings related to catheter use, the ability of various strains, including *E. faecalis* SY, to multiply in urine was assessed (*Vebø* et al., 2010). Division time did not vary much between *E. faecalis* SY and pathogenic strains, though the latter reached higher cell densities in urine ($2x10^8$ compared to $1.2x10^8$ CFU/ml for *E. faecalis* SY). Transcriptional analysis of bacteria exposed to urine for 30 minutes was performed by microarray analysis (the array did not contain *E. faecalis* SY-specific genes). The results indicated the bacteria had switched to alternative carbohydrate metabolism since glucose levels in urine are low, as well as other adaptations. In most of the described observations, *E. faecalis* SY behaved similar to the pathogenic strains MMH594 and OG1RF. This study was not conducted to specifically compare probiotic *E. faecalis* with virulent strains, but one observation was noticeable according to the authors: gene EF3314 was only upregulated in *E. faecalis* SY and not in the other two strains as a result of urine exposure. They described the product of EF3314 as a potential substrate for sortase A (coded by *srtA*, EF3056, also upregulated), which is important for adherence to abiotic surfaces and biofilm formation, and is considered a virulence gene for *E. faecalis* (*Vebø* et al., 2010). Comparative genome hybridization (CGH) was also performed to compare the three strains. As expected, variation was observed in mobile elements mainly. Of interest is also the observation that serotype 2 capsular polysaccharide genes (*cps*) were absent in the *E. faecalis* SY strain they used, which may explain why these bacteria are sensitive to phagocyte killing. From this observation it is likely they worked with S01. The MLST sequence type of *E. faecalis* SY was reported to be CC25, ST248, in accordance to the sequence type that Fritzenwanker and colleagues had obtained.

Hoffmann and colleagues investigated the ability of *E. faecalis* to cause colitis, using a knock-out mouse deficient of IL-10, which renders the animals susceptible to chronic intestinal inflammation in response to certain *E. faecalis* strains (*Hoffmann* et al., 2011). In a healthy host, the inflammatory response to bacteria present in the gut is tightly regulated so that non-pathogens are safely ignored (though monitored), but when this regulation is faulty, chronic inflammation is the unwanted outcome. A mouse model in which the 'safety valve' IL-10 is knocked out is used as a model to elucidate the causes of Crohn's disease (CD) and inflammatory bowel disease (IBD) in humans. When *E. faecalis* SY was compared *in vitro* to the colitogenic control strain OG1RF, both induced IL-6 and IFN-γ production in a non-differentiated murine cell line (*Hoffmann* et al., 2011). These findings are in line with those previously reported by *Rosenkranz* and *Grundmann* (1994). Both strains triggered pro-inflammatory responses via the pattern recognition receptor TLR-2 (*Hoffmann* et al., 2011). The probiotic bacteria also resulted in pro-inflammatory epithelial cell activation *in vivo*, but only when tested in germfree IL-10-deficient mice; wild-type animals did not develop disease in response to *E. faecalis* SY or the colitogenic strain (*Hoffmann* et al., 2011). It should be pointed out, that *E. faecalis* probiotics can reduce colitis symptoms in IL-10-deficient mice suffering from colitis when they are not raised germ-free. The authors admit that missing IL-10 is an immense immunological deficiency,

and that their mouse model is rather simplistic compared to human IBD.

Using a number of *in vitro* tests, Christoffersen and colleagues compared *E. faecalis* SY with four other *E. faecalis* strains: pathogenic V583 (the well-researched VanA-resistant clinical isolate), two commensal faecal isolates from babies (strains 62 and 158B) and a cheese strain called LMGT3208 (*Christoffersen* et al., 2012). After 30 min exposure to human gastric juice of pH 1.5, viability of all strains was severely impaired, and even when the pH was artificially raised to 5, exposure to the digestive enzymes present in gastric juice damaged all 5 strains. However, *E. faecalis* SY was least capable to tolerate acid exposure, as it grew poorly in BHI of pH 4.5 compared to the other strains. Exposure to human duodenal juice for 30 minutes also affected viability, but in this test *E. faecalis* SY survived better (91% survival) than the other four strains. Adherence to Caco-2 cells or mucin was significantly weaker for *E. faecalis* SY than for the others. Binding to glycosaminoglycans (host cell receptors) or inducing dendritic cell (DC) maturation was similar for all strains (*Christoffersen* et al., 2012). These results would suggest that *E. faecalis* SY is not well equipped to survive in the human stomach.

The capability to survive passage through the gastrointestinal tract was further assessed using two *in vitro* models to resemble passage through an empty stomach as well as a full stomach (*Wassenaar* et al., submitted for publication). Again, *E. faecalis* SY was rapidly inactivated by gastric pH, though sufficient bacteria survived to produce stable numbers in conditions subsequently resembling the small intestine. Nevertheless, a single dose did not result in intestinal colonisation, when tested with a single volunteer: live *E. faecalis* SY were only detected in stool for a few days following a single dose (*Wassenaar* et al., submitted for publication). This could be demonstrated with the use of strain-specific primers that had been designed by use of the genome sequence.

In view of the functionality of *E. faecalis* SY to treat recurrent upper respiratory tract infections and sinusitis, it was tested if the bacteria were able to colonise the throat instead. Two volunteers gurgled with a single dose of the product for 30 seconds, after which the product was swallowed. Immediately thereafter, live bacteria could be detected from swabs of the mouth and throat of both individuals, but not any more two hours later, or at any other time point tested up to 24 hrs. (C. Beimfort and K. Zimmermann, unpublished observations). It has not yet been established if prolonged exposure with multiple doses results in intestinal colonisation, or in elevated saliva IgA levels in humans.

## *ESCHERICHIA COLI* PREPARATIONS

### Animal studies with *E. coli* SY

The 1983 publication already cited for mouse experiments with *E. faecalis* SY also described how 4 weeks of oral administration of *E. coli* SY provided a similar level of protection against intraperitoneal challenge with *H. influenzae* (*Rusch* et al., 1983). Similar results were published a year later, when it was described that the $LD_{50}$ increased from an intraperitoneal dose requiring a $10^{-6.0}$ dilution of challenge *H. influenzae* (no probioticum) to a dilution of $10^{-3.8}$ when the challenge followed 3 weeks of *E. coli* administration (*Rusch* et al., 1984).

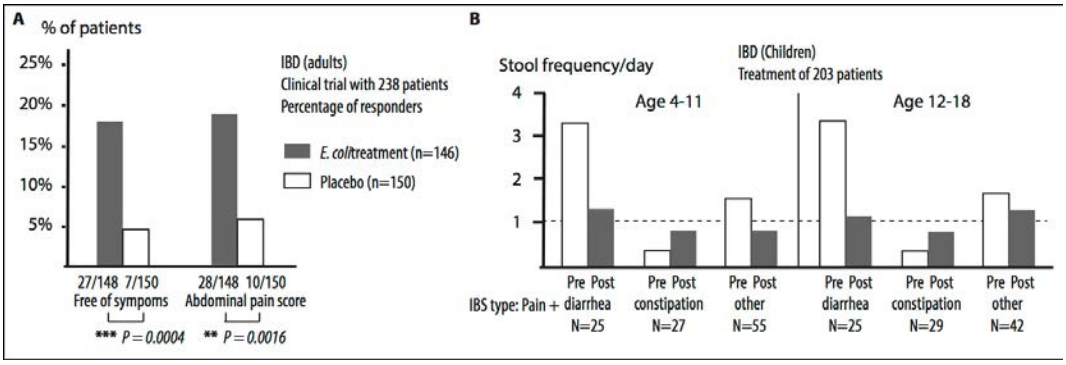


**Figure 6:** Treatment of IBD with *E. coli* SY.
Panel A shows the key outcome of a clinical trial involving 238 adult IBD patients. (After *Enck* et al., 2009). Panel B shows improved stool frequency in two age groups of paediatric IBD patients. (Modified after *Martens* et al., 2010).

As with *E. faecalis*, the mode of action of probiotic *E. coli* is most likely via immune-modulation. A key publication in this respect is from 2008, which describes the function of mast cells as the policemen of the immune system (*Magerl* et al., 2008). When these cells encounter pathogenic bacteria, they release pre-synthesized substances to recruit lymphocytes and dendritic cells, whereby they respond differently to non-pathogenic bacteria. In response to the latter, mast cells produce IL-15, which tunes down the recruitment of immune cells, together with a collection of other genes that are up- or down regulated during the process. An *in vitro* model using murine mast cells was applied to investigate how these cells respond to *E. coli* SY (*Magerl* et al., 2008). When pre-incubated with the bacteria, the mast cells were no longer triggered by a calcium ionosphere or an IgG/allergen combination. The observed inhibition of degranulation was concentration-dependent in a binary manner, with little effect observed below 15,000 bacteria per mast cell, but no further increase at higher inoculates. Sterile culture supernatant or paraformaldehyde-killed bacteria did not inhibit degranulation. When mice were injected intraperitoneally with a suspension of the probiotic bacteria, mast cells harvested a day later from the peritoneum were again less responsive to the tested triggers. By varying the time between *i.p.* dosage and mast cell harvest, it was established that the effect faded over the course of a few days (*Magerl* et al., 2008). As a side note, it should be mentioned that the mice did not suffer from the bacterial load and survived the treatment till the end of the experiments.

## Clinical trials with *E. coli* SY

A randomized double-blind clinical trial was published in which *E. coli* SY was tested for treatment of IBD, involving 238 patients. Treatment lasted for 8 weeks, The key finding was that there were significantly more responders that became free of symptoms (27 out of 148) than in the placebo group (7/150) (*Enck* et al., 2009). The percentages for patients with lower abdominal pain scores were also significantly different. The results are summarized in Figure 6, Panel A. The trial was included in a systematic review that was performed to assess probiotic therapies in adult IBS patients, which covered 37 publications in total (*Hungin* et al., 2013). The majority of

the compared studies focused on IBS with diarrhoeal symptoms. The results obtained with *E. coli* SY were cited in 5 of 16 statements by Hungin and colleagues: nr. 1, [the product] help(s) relieve overall symptom burden; nr. 4, helps reduce abdominal pain; nr. 5, helps reduce bloating/distension; nr. 8, helps improve frequency and/or consistency of bowel movements; and nr. 12, improvement of symptoms leads to improvement in some aspects of health-related quality of life (*Hungin* et al., 2013).

Treatment with *E. coli* SY was also tested in 203 paediatric IBD patients (age range 4-11 and 12-18 years), although this was not a blinded or placebo-controlled study (*Martens* et al., 2010). The children were treated for 43 days on average and this treatment was well tolerated. The stool frequency improved, both for IBD in combination with diarrhoea as for constipated patients (Figure 6B). Pain also decreased significantly in both age groups that were analysed.

**Mechanistic explanations**

Early work by Jansen and co-workers had shown how intake of *E. coli* SY changed levels of immunoglobulins (*Jansen* et al., 1998). Ten healthy volunteers donated two serum samples (3 weeks apart) prior to a 2-weeks daily intake of the product, after which another serum sample was taken, with a follow-up 4 weeks later. Faecal samples were collected on a weekly basis during the complete investigation. Serum IgG, IgM and IgA levels were tested for binding capacity to *E. coli* SY, whereby the individual's levels prior to intake of the product served as an internal control. This work demonstrated an increase in IgG serum levels only, for all 10 individuals, that lasted throughout the follow-up period (*Jansen* et al., 1998).

That *E. coli* induces production of epithelial β-defensins in the human host provides a mechanistic explanation of beneficial effects of *E. coli* SY. After 23 healthy volunteers had taken the product for 3 weeks, their stool samples contained elevated levels of human β-defensin-2 (hBD-2), as determined by ELISA, in contrast to 5 volunteers taking placebo (*Möndel* et al., 2009).

The ability to inhibit the growth of pathogenic species, as in the originally proposed mechanism of probiotic *E. coli* by Alfred Nissle, was investigated by comparing *E. coli* Nissle 1917 and a number of commensal isolates for their ability to inhibit growth of Shiga-toxin producing *E. coli* (STEC) (*Reissbrodt* et al., 2009). In comparison to *E. coli* Nissle and a serendipitously found commensal strain, which both reduced Shiga toxin levels with over 90% during co-cultivation, *E. coli* SY could only reduce these levels with 10%, whereas other strains and *E. faecalis* SY bacteria had no effect at all (*Reissbrodt* et al., 2009).

Novel insights on the mechanism of probiotic action were obtained with the discovery of Microcin S (*Zschüttig* et al., 2012). This novel type of bacteriocin is only produced by *E. coli* SY genotype G3/10, transcribed from an operon of 4 genes present on the megaplasmid of this isolate. *In vitro* activity of the microsin against an enteropathogenic *E. coli* strain (EPEC) was demonstrated, and the authors speculated on the use of this novel bacteriocin as a potential antitumor agent (*Zschüttig* et al., 2012). According to the scheme of Cotter and colleagues, Microcin S belongs to class IId (unmodified anacyclamides) (*Cotter* et al., 2013).

Zschüttig and colleagues speculated on the use of this novel bacteriocin as a potential antitumor agent. The idea was

further investigated in a recent publication (*Kocijancic* et al., 2016). In mouse experiments it was demonstrated that the Lux-marked G1/2 strain of *E. coli* SY specifically target tumours after intravenous administration. Whereas bacteria rapidly reached liver and spleen, from which they were cleared, tumours were colonised more slowly but persistently (*Kocijancic* et al., 2016). This line of work could open future perspectives of the product as a delivery vehicle for anti-tumour therapeutics.

**Strain characterization and genome sequences of *E. coli* SY**

The 10 subcultures that make up *E. coli* SY can be divided into 4 or 6 genotypes, depending on the sensitivity of the typing method applied. These are called G1/2 (20% in the final product), G3/10 (20%), G4/9 (20%), G6,7 (20%), G5 (10%), and G8 (10%). The strains G1/2, G6/7 and G8 contain the same two plasmids and share a number of other characteristics (see below), so that they are sometimes regarded as representatives of one and the same genotype. All originate from the stool of a healthy volunteer.

The first genetic characterisation was performed by microarray analysis, using a microarray based on 24 *E. coli* and 8 *Shigella* genome sequences that were available at that time (*Willenbrock* et al., 2007). The analysis was performed with G1/2, G3/10, G4/9 and G5 (G6/7 and G8 were omitted), resulting in the first detailed insights about the genetic make-up of these types. The number of predicted genes varied from 3568 (G4/9) to 3978 (G1/2), and the four genotypes shared marginally more genes with commensal strain *E. coli* K-12 MG 1655 than with enteropathogenic strain EDL933. A hierarchical cluster analysis of the hybridization signals revealed that the genomes of the *E. coli* SY components more closely resembled K12 than EDL933 or other pathogenic strains. A core genome of 3083 genes was identified that all four genotypes shared (*Willenbrock* et al., 2007). The authors further reported presence of a haemolysin operon (*hlyABCD*) in genotype G1/2, in accordance to the weakly haemolytic phenotype of this isolate. Genotypes G6/7 and G8 also contain this operon.

The genome sequences of the 6 genotypes were released in 2015, accompanied by a genome announcement publication (*Zschüttig* et al., 2015). The chromosome of G3/10 was assembled to completion, while the other genotypes were published as multiple contigs. The plasmids of the strains, varying in number from 1 (G4/9) to 6 (G3/10) were also sequenced.

**Safety and colonisation aspects of *E. coli* SY**

The haemolysin gene was not the only virulence gene detected in these strains; other genes that are typically associated with pathogenic *E. coli* were found present as well. These were described and discussed in a publication that concentrated on the safety of the product (*Wassenaar* et al., 2015). It was concluded that presence of individual virulence genes cannot be taken as evidence for pathogenicity per se. Combined with the ten-years long collation of all side effects collected from commercial use, which was a surprisingly short list, it was concluded that the product was safe for human use (*Wassenaar* et al., 2015). This conclusion was further substantiated in a discussion paper where evidence from other *E. coli* strains was also included, to support the view that presence of virulence genes is not always a sign of a pathogenic lifestyle: the genomic background determines to a large extent the phenotype of a strain of *E. coli*

(*Wassenaar* and *Gunzer*, 2015).

The genome sequences were used to develop strain-specific probes, which enabled the specific detection of *E. coli* SY components. These were used to determine the colonisation potential of the product in humans after a single dose. (*Wassenaar* et al., 2014). It turned out that the strains colonised well: all volunteers were colonised by *E. coli* SY for at least 12 weeks and for two individuals the product could be detected for 27 weeks. This was demonstrated by colony-lift hybridization from stool cultures. During the first week following the single dose intake, the detected genotypes fluctuated, but after a few weeks all but one of the genotypes had disappeared. In all volunteers only genotype G1/2, which could not be distinguished from G6/7 and G8, survived (*Wassenaar* et al., 2014). Not all volunteers took the same dose, and by comparison of the highest and lowest dose, which were a factor of 10 apart, it was deduced that the persistence of these three genotypes (which together comprise 50% of the product) was not the result of a numerical advantage in the dose.

By use of dynamic *in vitro* models it was established that *E. coli* SY were decimated during gastric passage, but enough bacteria survived to multiply as soon as conditions mimicking the jejunum were applied (*Wassenaar* et al., submitted for publication). It has not yet been established if dominant colonisation of the genotype G1/2 would also result if this strain was administered by itself, or whether an interplay with the other genotypes creates a local niche, which subsequently enhances growth of G1/2.

## BACTERIAL LYSATE PSY

### Animal studies with the bacterial Lysate PSY

The effectivity of Lysate PSY to alleviate immunological overreaction was first modelled for food allergy by use of a rat model (*Ahrens* et al., 2011). Rats were sensitized with intraperitoneal ovalbumin (OVA) to trigger an allergic reaction, after which the animals were orally challenged with OVA, and humoral antibody levels were determined. One group of animals received Lysate PSY prior to and during sensitization, while the control group did not receive the lysate. Sensitization resulted in a 75% increase of total IgE levels in both groups, but while OVA-specific IgE was absent in non-sensitized animals, these levels were much lower in the treated animals than in the control group; similar results were recorded for IgG (*Ahrens* et al., 2011). Mononuclear cells (MNCs) in the spleen were isolated post-mortem and these were stimulated with OVA *in vitro*. The cells from sensitized animals responded stronger than cells from non-sensitized animals, but not when the animals had been treated with the bacterial lysate. When the cytokines produced by spleen MNCs were compared, the cells isolated from the treated rats produced significantly more IL-10 than the cells isolated from control animals. An even stronger difference was seen with MNCs isolated from the mesenteric lymph nodes. All these results suggested that the treatment with Lysate PSY had dampened the immune response to the ovalbumin allergen ovalbumin (*Ahrens* et al., 2011).

### Clinical studies with Lysate PSY

A clinical trial that was performed with Lysate PSY in 1988-1989 was originally published in German (*Panijel* and *Burkhard*, 1993) and later re-analysed

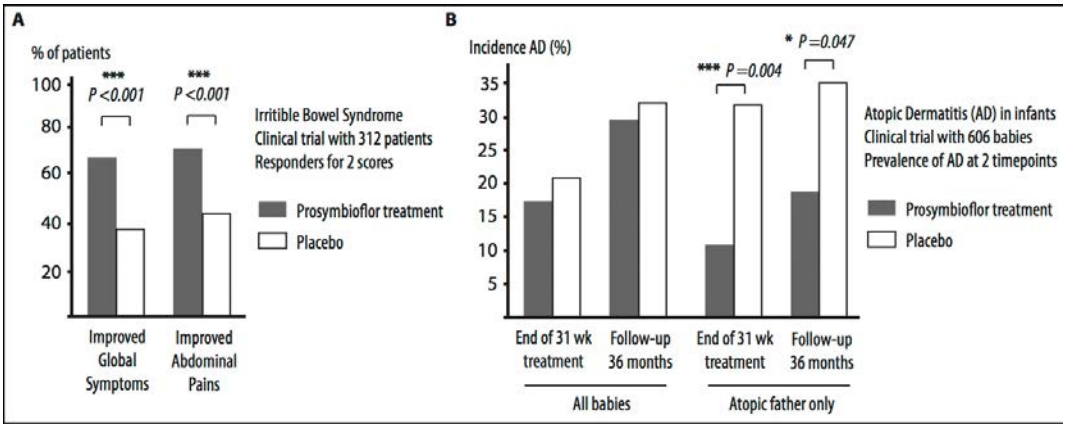


**Figure 7:** Results from two clinical trials with bacterial Lysate PSY.
Panel A shows the percentage of responders in a clinical trial involving 312 adult IBS patients who were treated for 8 weeks, based on two scores. (Modified after *Enck* et al., 2008). Panel B shows a trial with 606 babies with atopic dermatitis (AD) as the measured outcome, determined after 31 weeks of treatment and at a follow-up time-point of 36 months. There was a significant difference only for a subgroup of patients whose father had a history of atopic disease. (After *Lau* et al., 2012).

and presented in English (*Enck* et al.,2008). The reason for the re-analysis was that when the trial was originally described, neither the Rome criteria for the diagnosis of IBS, nor the FDA and EMA guidelines for analysis of clinical trials had been available, which were subsequently applied. The double-blinded trial involved 312 adult IBS patients who suffered from abdominal pain. Of these, 297 were randomly divided into a placebo and treatment group. Treatment lasted for 8 weeks. During the trial 18 patients dropped out, 5 (3 in the treatment and 2 in the placebo group) because of adverse effects. The outcome of treatment was recorded using a global symptom score (GGS) and an abdominal pain score. Based on the GGS, 102 of the 149 patients (68.5%) receiving Lysate PSY were considered as drug responders, meaning the symptom score had significantly decreased. This was a highly significant difference compared to the 37.8% responders receiving placebo, visualized in Figure 7, Panel A. A similar positive result was obtained for abdominal pain scores, with 72.5% responders in the treatment and only 44.6% in the placebo group. The authors concluded that although there is no mechanistic explanation how bacterial lysates can relieve symptoms in IBS patients, such products may have lower adverse events and a higher acceptance in patients compared to classical probiotic products based on live bacteria (*Enck* et al., 2008).

A meta-analysis published a year later included the results of the Enck study, though it cited a meeting abstract as the source, because the complete publication had not yet been available when the meta-analysis was performed (*McFarland* and *Dublin*, 2008). In the resulting forest plot, the Enck study showed a favourable effect with an relative risk of 0.51 (95% confidence interval 0.39-0.66) of relative risk for IBS symptoms after probiotic treatment compared to placebo. Note that in this way of expressing the data, an relative risk <1 is indicative of a therapeutic effect. With this score, Lysate PSY was amongst the better-

producing microbiologics, while the findings had a small 95% confidence interval.

Another clinical trial was directed towards prevention of atopic dermatitis in children (*Lau* et al., 2012). The double-blind, randomized placebo-controlled trial involved infants with one or two atopic parents as well as children without such a genetic history. A total of 606 babies were enrolled, half of who received Lysate PSY from week 5 till 7 months of age. The children were followed until they reached the age of 3 (*Lau* et al., 2012). The number of children not completing the treatment, as well as number and severity of adverse effects, were not significantly different between treatment and control group. Disappointingly, when the complete study group was analysed, treatment and control group apparently were apart, but they did not significantly differ in frequency of atopic dermatitis (Figure 7B). This was also true for the three subgroups of children who had two atopic parents, children with parents who were both not atopic, and children with an atopic mother (*Lau* et al., 2012). However, the prevalence of atopic dermatitis was significantly lower ($p<0.004$) in the treatment group compared with controls for children whose father but not the mother was atopic. This difference was obvious at the end of the intervention phase (week 31) but tailed off to a borderline significance ($p<0.047$) at 36 months. The authors stated they were aware that subgroup analysis in a clinical trial must be interpreted with caution. One should not analyse towards a desired effect, but in this case it was known that prevalence of atopic dermatitis is in part related to the atopic state of the parent(s). Although the activity of LPS in the gut can serve as a mechanistic explanation, the difference in protection for atopic fathers versus no protection for atopic mothers cannot be explained. Before speculating on underlying mechanisms at play here, more data would be needed; not only on the mechanism of action of Lysate PSY but also a confirmation of the trial findings with an independent study.

A year later, the topic was revisited by a new analysis of the same infants cohort. During the trial, stool samples of the children had been collected at week 5 (when the Lysate PSY intervention started), at week 13 and at week 31, when intake of the lysate was terminated. The microbiome of these stool samples were investigated by culture-dependent methods and by DNA-dependent methods. (*Penders* et al., 2013). The results provided some valuable insights in the composition of the microbiota of an infant. First of all, whether the babies had received Lysate PSY or placebo made no difference, which was to be expected, since the lysate does not contain viable bacteria. This observation rules out the possibility that any clinical effect of Lysate PSY is the indirect result in shifts of intestinal microbiota (in theory, the microbiota could shift as a result of the bacterial lysate, or by induced local immune-modulation, but neither seemed to be the case). However, as had been observed before, birth mode (vaginal versus caesarean birth) had a profound effect on microbiota composition (*Penders* et al., 2013). Surprisingly, another independent and strong influence was found for birth order: with increasing number of older siblings, the number of *Lactobacillus* and *Bacteroides* species increased, while frequency of Clostridiales decreased (*Penders* et al., 2013). Especially this later finding is relevant, as the risk of developing atopic dermatosis increases with *Clostridium* colonisation, and this was found in the cohort under study as well, in particular for members of *Clos-*

*tridium* cluster I. No effect on composition of the intestinal composition was reported for day care attendance or maternal and paternal atopy.

This is not to say that early administration of Lysate PSY can prevent other atopic symptoms. The same study groups were analysed at age 6-11 years for prevalence of asthma or rhinoconjunctivitis, for which no significant difference between treatment and placebo group could be established (*Rossberg* and colleagues, unpublished data).

The economic burden of atopic dermatitis and the cost-effectiveness of possible prevention by Lysate PSY were calculated by *Kiencke* et al. (2013). It was calculated that preventive treatment with Lysate PSY was cost effective at 3 years and at 6 years for children with one atopic parent ('single heredity for atopy'). The effectiveness of costs would probably be even higher for children with an atopic father.

**Product characterization and quality control of Lysate PSY**

Lysate PSY is produced by separate culture of the bacterial components *E. faecalis* SY and *E. coli* SY to approximately $3x10^7$ CFU/ml, after which the bacterial cultures are cooled to 6˚C. The cultures are then combined and sterilized at 121˚C for 20 minutes under constant pressure. The result is a clear solution, which is bottled in a sterile environment.

Concerns about variable content and lack of quality control of a bacterial lysate were minimised by a study by Klein and co-workers, who used a proteomics approach to determine the quality of Lysate PSY (*Klein* et al., 2013). By analysis of the total protein content, the complexity of the components present in a bacterial lysate can be assessed qualitatively as well as quantitatively. The authors used a high-throughput, high-resolution technique in which capillary electrophoresis was coupled to mass spectrometry (CE/MS). The output is presented in the form of three-dimensional contour plots. These capture the migration time of electrophoresis, the mass (on a logarithmic scale) of detected peptides in kDa and the signal intensity as a quantitative measure (*Klein* et al., 2013). The results showed that various lots of Lysate PSY were of relatively constant qualitative constitution, although the data would have been more convincing if a deliberate aberrant sample (e.g. the separate lysates of *E. faecalis* and *E. coli*) were also included for comparison. The major differences reported between lots were quantitative, as some individual peak heights varied between batches (*Klein* et al., 2013). It must be noted, that peptides were also detected in sterile culture media, though there were 3 times more different peptides found in the bacterial lysate. The major peptides were recovered and sequenced, which identified that 517 peaks were derived form *E. faecalis* proteins and 406 were from *E. coli* (*Klein* et al., 2013). Interestingly, whereas the majority of peptides from *E. coli* proteins originated from cell membrane and fimbriae, those of *E. faecalis* were mostly cytosolic.

## CONCLUSIONS

The data presented in this paper allow the conclusion that symbiotic bacteria offer a great therapeutic potential. Furthermore the results of multidisciplinary investigations around bacterial products based upon Symbio *E. fae-*

*calis* SY and *E. coli* SY open possibilities to hitherto unexpected medical targets. This will require many more research efforts. There is no doubt, however, that the use of microbiologic or probiotic drugs containing live *Enterococcus faecalis* or live *Escherichia coli* or a bacterial lysate thereof is safe. In numerous experimental *in vitro* and animal studies, as well as by human clinical studies, efficacy of such microbiologics or probiotics was established. The mode of action is apparent in modulation of many physiologic parameters and most prominently in modulation of immune activities.

## IDENTIFICATION OF BACTERIAL PRODUCTS DESCRIBED IN THIS PUBLICATION

The bacterial preparations described in this paper are produced by SymbioPharm GmbH, Herborn, Germany. We use the following abbreviations for the products of SymbioPharm: *E. faecalis* SY describes Symbioflor 1 containing live *Enterococcus faecalis (DSM 16440)*, *E. coli* SY is used for Symbioflor 2 containing live *Escherichia coli (DSM 17252)*, and Lysate PSY describes Pro-Symbioflor, a bacterial lysate manufactured from *Enterococcus faecalis (DSM 16440)* and *Escherichia coli (DSM 17252)*.

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