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# Prophage-mediated modulation of interaction of *Streptococcus thermophilus* J34 with human intestinal epithelial cells and its competition against human pathogens

**C. Guigas[1*], K. Faulhaber[2], D. Duerbeck[3], H. Neve[1] and K.J. Heller[1]**

*[1]Department of Microbiology and Biotechnology, Max Rubner Institut (Federal Research Institute of Nutrition and Health), Hermann-Weigmann-Straße 1, 24103 Kiel, Germany; [2]Chair Organismic Interactions, Interfaculty Institute of Microbiology and Infection Medicine, Tübingen University, Geschwister-Scholl-Platz, 72074 Tübingen, Germany; [3]BAV-Institut für Hygiene und Qualitätssicherung, Hanns-Martin-Schleyer-Str. 25, 77656 Offenburg, Germany; claudia.guigas@mri.bund.de*

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**RESEARCH ARTICLE**

## Abstract

The human intestinal microbiota plays an important role in human health. While adhesion to gastrointestinal mucosa is a prerequisite for colonisation, inhibition of adhesion is a property which may prevent or reduce infections by food borne pathogens. *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* represent the two lactic bacteria constituting the yoghurt culture. These starter cultures have been claimed to be probiotic. In our study we compared two *S. thermophilus* strains (i.e. lysogenic strain J34 and corresponding non-lysogenic [prophage-cured] strain J34-6), with respect to (1) their *in vitro* adhesion properties to HT29 cells and (2) their cell surface hydrophobicities. Effects of the two strains on inhibition of adhesion of the pathogens *Listeria monocytogenes* Scott A, *Staphylococcus aureus* 6732 and *Salmonella enteritidis* S489 were studied *in vitro* with HT29 cell cultures. Lysogenic strain J34 was shown to be considerably more effective than the non-lysogenic derivative strain J34-6.

**Keywords:** lactic acid bacteria, *Streptococcus thermophilus*, *in vitro* adhesion, human pathogens

## 1. Introduction

Yoghurt is a fermented dairy product, which is considered as a particularly healthy food. Thus, yoghurt, the yoghurt culture per se or the two lactic acid bacteria *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* constituting the starter culture have often been proposed to be probiotic (Guarner *et al.*, 2005). EFSA in 2010 accepted the health claim 'lactose digestion' for 'live yoghurt cultures' (EFSA, 2010). According to the International Scientific Association for Probiotics and Prebiotics (ISAPP) probiotic mechanisms are classified from rare (strain specific such as immunomodulatory effects) over frequent (species-level properties such as bile salt metabolisms) to widespread ones, such as competitive exclusion of pathogens (Hill *et al.*, 2014). This definition also takes into account the generally accepted view that probiotic properties are strain specific and that probiotic properties – i.e. health benefits – have to be demonstrated unambiguously by appropriate experimental studies.

Collado *et al.* (2008) tested the effects of different strains of lactic acid bacteria and bifidobacteria on adsorption of *Enterobacter sakazakii* to mucus and reported that an *S. thermophilus* strain was (among other strains) effective in preventing of *E. sakazakii* adsorption. Whether those experiments have any relevance for *in vivo* health effects remains to be demonstrated.

*S. thermophilus* strain J34 has been isolated from a yoghurt sample in Germany and contains the small cryptic 3.383-kb plasmid pJ34 (Geis *et al.*, 2003). J34 is a lysogenic strain harbouring prophage phage TP-J34 (Neve *et al.*, 1998, 2003). A lipoprotein determinant (*ltp*) was identified on the phage TP-J34 genome upstream of the phage integrase gene, which is also present in a few closely related *S. thermophilus* phages (Ali *et al.*, 2014). Ltp, which is expressed during lysogeny, is exposed on the outer surface of the cytoplasmic membrane where it interferes with DNA injection of the homologous phage and of some heterologous phages (Sun, 2002; Sun *et al.*, 2006). Prophage TP-J34 thus causes

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'lysogenic conversion' by conferring a 'superinfection exclusion' (*sie*) phenotype (McGrath *et al.*, 2002) onto the lysogenic *S. thermophilus* J34 host strain.

*S. thermophilus* J34 grows homogeneously in liquid culture. However, when the prophage was cured from the strain, the growth behaviour of the prophage-cured *S. thermophilus* strain J34-6 changed drastically and the J34-6 cells aggregated intensively (Neve *et al.*, 2003). Ltp, known to be a surface-exposed protein, was not involved in the notably different growth behaviours: knocking out expression of *ltp* by integration of a plasmid harbouring an internal *ltp* DNA fragment did not affect the phenotype of homogeneous growth (Sun, 2002). So far, the mechanisms altering growth behaviour and the surface structures involved are not known.

Walter *et al.* (2008) demonstrated that auto-aggregation of a *Lactobacillus reuteri* strain appeared to be an important aspect for colonisation. This prompted us to compare the two *S. thermophilus* strains J34 and J34-6, differing in their aggregation phenotypes, with respect to their adhesion properties to intestinal cells and their potential to interfere with adhesion of known bacterial pathogens.

The human intestinal microbiota plays a pivotal role in human health. While adhesion is a prerequisite for microbial colonisation of gastrointestinal mucosa, inhibition of adhesion is a property which may help to prevent or reduce infections by food borne pathogens. By protecting the host from colonisation with enteropathogens (i.e. by barrier effects), probiotic bacteria might play a role in defence and recovery from enteropathogen infections (Candela *et al.*, 2008; Servin *et al.*, 2004). Selected probiotic bacterial strains should be able to compete with pathogens for the same receptors and occupy their potential binding sites in the gut (Lorca *et al.*, 2002; Styriak *et al.*, 2003).

In this study we identified a gene (*orf60*) of the 'lysogenic conversion' module of phage TP-J34 (Brüssow and Desiere, 2001) as being involved in homogeneous growth of lysogenic *S. thermophilus* J34. In addition, cell surface hydrophobicity of the two *S. thermophilus* strains J34 and J34-6 was determined. Effects of the two strains on inhibition of adhesion of the pathogens *Listeria monocytogenes* Scott A, *Staphylococcus aureus* 6732 and *Salmonella enteritidis* S489 were studied *in vitro* using the human HT 29 cell line.

## 2. Materials and methods

### Cell culture and bacterial strains

HT29 human colon adenocarcinoma epithelial cells (DSMZ no. ACC 299) were routinely grown in antibiotic-free Dulbecco's modified Eagle's essential medium supplemented with 10% foetal calf serum (FCS) (Invitrogen, Eggenstein, Germany) at 37 °C in a 10% $CO_2$ atmosphere.

The bacteria applied in this study are listed in Table 1.

*S. thermophilus* strains were routinely grown in M17-bouillon (Merck, Darmstadt, Germany) according to Terzaghi and Sandine (1975) at 37 °C. The following pathogenic strains were used: *S. aureus* 6732 (DSMZ, Braunschweig, Germany), *S. enteritidis* S489 and *Listeria monocytogenes* Scott A (both strains were from the culture collection of MRI, Karlsruhe, Germany). They were selected due to their roles as pathogens for humans and their presence in the human gut. Pathogenic strains were routinely grown in Standard I nutrient broth (Merck) at 37 °C. For adhesion studies, overnight-cultures of *S. thermophilus* and pathogens were harvested by centrifugation at 10,000×*g* for 5 min, washed twice with sterile phosphate-buffered saline (PBS, pH 7.3) and resuspended in Dulbecco's modified Eagle's minimal essential medium (DMEM; Invitrogen, Eggenstein, Germany). Cell count was determined using

**Table 1. Bacterial strains applied in this study.**

| Bacterial strain | Genotype | Reference/Source |
|---|---|---|
| *Streptococcus thermophilus* J34 | lysogenic wild type carrying prophage TP-J34 and plasmid pJ34 | Neve *et al.*, 2003; Geis *et al.*, 2003 |
| *S. thermophilus* J34f-2 | *S. thermophilus* J34 cured of plasmid pJ34 | Sun, 2002 |
| *S. thermophilus* J34-6 | *S. thermophilus* J34 cured of prophage TP-J34 | Neve *et al.*, 2003 |
| *S. thermophilus* J34-12f | *S. thermophilus* J34 with non-inducible prophage TP-J34 (due to mutation in *rir*) and cured of pJ34 | This study; Neve *et al.*, 2003; Koberg *et al.*, 2015 |
| *S. thermophilus* DD04g-int10 | *S. thermophilus* J34-12f with pGhost9:IS*S1* inserted into *orf60* of prophage TP-J34 | Maguin *et al.*, 1996 |
| *S. thermophilus* J34f-2-KOorf60 | *S. thermophilus* J34f-2 with plasmid pKFKOorf60 inserted in *orf60* of prophage TP-J34 | This study |
| *Salmonella enteritidis* S489 | | Max Rubner-Institut, Karlsruhe, Germany |
| *Staphylococcus aureus* 6732 | | DSMZ, Braunschweig, Germany |
| *Listeria monocytogenes* Scott A | | Max Rubner-Institut, Karlsruhe, Germany |

a flow cytometer (IUL, Königswinter, Germany). The mean value from 20 determinations was used to prepare the inocula (adjusted with DMEM) for the adhesion test (approximately $1\times10^8$ cfu/ml for the three pathogens and $2\text{-}3\times10^8$ cfu/ml for *S. thermophilus* strains). The *Streptococcus*/pathogen ratio of 3:1 was based on studies of Xu *et al.* (2009), who found for Caco-2 adhesion rates exceeding 12% in the case of *S. aureus* at an inoculum of $1\times10^8$ cfu/ml, and of Conway *et al.* (1987), who found that *S. thermophilus* adhered poorly to human intestinal cells. For determination of inocula serial dilutions were plated on M17 (*S. thermophilus*), Standard I nutrient (*S. aureus* and *S. enteritidis* ) and Palcam agar (Van Netten *et al.*, 1989), respectively, and incubated at 37 °C. *S. thermophilus* was incubated at anaerobic condition at 42 °C. When necessary, erythromycin (Em) was used in concentrations of 1 and 2 µg/ml for liquid and solid media, respectively.

For cloning of pGhost-derivatives, *Escherichia coli* EC1000 (kanamycin-resistant, pWV01 repA+ integrated in *glgB* (Leenhouts *et al.*, 1996; Sanders *et al.*, 1995) was applied.

### *In vitro* adherence assay for *Streptococcus thermophilus* strains and pathogens

Totals of $4\times10^5$ HT29 cells in 1 ml DMEM were seeded in 24-well tissue plates (Greiner Bio-One, Frickenhausen, Germany) 24 h prior to infection. After centrifugation, washing (PBS), counting, and resuspension in DMEM, 1 ml aliquots of bacterial suspensions were added to each well of the tissue culture plates, plates were centrifuged at 2,000×*g* for 2 min and incubated in a 10% $CO_2$ atmosphere. At the same time, viable counts of the inocula of *S. thermophilus* and pathogens were determined by plating serial dilutions on M17-, Standard I nutrient- (*S. aureus*, *S. enteritidis*) and Palcam-agar (*L. monocytogenes*), respectively. After 1 h of incubation at 37 °C, cells were lysed by addition of Triton ×100 (0.05% solution) and viable counts of the adhered bacteria were determined by plating serial dilutions on M17-, Standard I nutrient- and Palcam-agar respectively. Adhesion was calculated from the initial viable counts and those of the cell lysates. Each determination was carried out in triplicate.

After determination of *in vitro* adhesion rates of streptococci and pathogens per se, *S. thermophilus* strains were added to HT29 enterocytes either simultaneously with (for competition) or 30 min before (for exclusion) or 30 min after (for displacement) pathogenic strains *S. aureus* 6732, *S. enteritidis* S489 or *L. monocytogenes* Scott A, respectively. For each test, *Streptococcus*/pathogen ratio was 3:1 (see above). After being released from enterocytes, the numbers of adhered pathogens were quantified by plate counting. Studying the susceptibility of *S. thermophilus* to antibiotics Tosi *et al.* (2007) reported minimal inhibitory concentrations (MICs) up to 16 µg/ml for gentamycin and 0.5 µg/ml for ampicillin. For determination of *S. aureus,* Standard I nutrient-agar plates with 0.5 µg/ml ampicillin were used, whereas *S. enteritidis* counting was done in Standard I nutrient-agar plates with 16 µg/ml gentamycin. Determination of *L. monocytogenes* was done on Palcam-agar. Each test was done in triplicate.

The inhibitory effect of the two *S. thermophilus* strains against the pathogens was calculated as follows:

$$Ie\ [\%]=100\times(1-T1/T2)$$

where T1 is the pathogen adhesion rate in the presence of *S. thermophilus* culture and T2 the adhesion rate in the absence of these cultures.

### Determination of cell surface hydrophobicity

The test for bacterial adhesion test to hydrocarbons (BATH; Doyle and Rosenberg, 1995) was adopted to screen cultures of *S. thermophilus* and pathogens for cell surface hydrophobicity. Bacteria were grown in appropriate broth at 37 °C for 18 h. Cells from 5 ml of culture were collected by centrifugation at 9,500×*g* at 4 °C for 6 min. Cells were washed twice with quarter-strength Ringer's solution (QSRS). One ml of this suspension was used to determine the $OD_{580}$ (reading 1). 1.5 ml of the cell suspensions were added to an equal volume of *n*-hexadecane as organic phase (Merck, Darmstadt, Germany) and thoroughly mixed for 2 min on a vortex. The phases were allowed to separate at room temperature for 30 min. Subsequently, 1 ml of the watery phase was used for measurement of turbity at $OD_{580}$ (reading 2). Calculation of hydrophobicity was done as follows:

% Hydrophobicity=

$$100\times\frac{(OD_{580}\text{ reading 1}-OD_{580}\text{ reading 2})}{OD_{580}\text{ reading 1}}$$

Hydrophobicity determinations were carried out in three replicates.

### Mutagenesis of *Streptococcus thermophilus J34-12f* by random insertion of pG$^+$host9-ISS1

For the knock-out experiments, the pJ34-cured, non-inducible lysogenic *S. thermophilus* strain J34-12f (exhibiting homogeneous growth behaviour (Neve *et al.*, 2003) was selected, as the TP-J34 prophage was stably inserted in the chromosome of this strain avoiding the risk of spontaneous phage induction, and pJ34 was cured for avoiding inference with pG$^+$host9-*ISS*1 transformation. This strain was transformed with pG$^+$host9-*ISS*1 (Maguin *et al.*, 1996), harbouring an Em resistance gene and expressing a temperature-sensitive replication protein, and subsequently grown at 30 °C in M17 medium with

2 µg/ml Em, to allow for replication of the plasmid. For integration of the plasmid, growth temperature was raised to 40 °C (i.e. the non-permissive temperature for pG$^+$host9-*IS*S1 replication). After growth to early stationary phase, the culture was left for 16 h at room temperature without shaking. J34-12f cells taken from the sediment of the first culture were inoculated and incubated at 40 °C to early stationary phase. A third subsequent culture was prepared under the same growth conditions to increase the number of cells with sedimenting phenotype. Finally, serial dilutions were made from the last enrichment culture and spread on M17 agar containing 1 µg/ml Em and incubated at 40 °C and Em-resistant colonies grown were tested for a sedimenting phenotype. For characterisation of the sites of integration according to Maguin *et al.* (1996) total genomic DNA was isolated and subjected to hydrolysis with HindIII and EcoRI, respectively. The restriction digests were diluted three to five-fold, to allow for intra-molecular ligation. After ligation and transformation, plasmids were isolated from colonies grown at permissive temperature. The regions flanking the sites of ligation were determined by DNA sequencing of plasmids using sequencing primers PGhISEco (5'-CCAACAGCGACAATAATCAC-3') and PGhISHin (5'-GAACCGAAGAAATGGAACGC-3'), respectively. DNA sequences were determined at Eurofins MWG Operon GmbH (Ebersberg, Germany).

### Targeted knock-out of orf60 in prophage TP-J34

For a targeted knock-out of *orf60*, we cloned an internal 273 bp fragment of *orf60* (total length: 420 bp) in pG$^+$host9 (Maguin *et al.*, 1996), yielding pKFKOorf60. The fragment was generated from isolated TP-J34 DNA by PCR with primers TP-J34orf60R (3'-TAAGAATTCAATCTCTTCGGAAGTGTAGTA-5') and TP-J34orf60HF (3'-GAAAATAAGCTTGG-TATCAGTCGTTCT-5'). Restriction sites for EcoRI and HindIII, respectively, which are underlined in the sequences, were used for cloning of the PCR product in *E. coli* EC1000. After (1) transformation of pKFKOorf60 into lysogenic and plasmid-free *S. thermophilus* J34f-2 (we used this strain to avoid eventual interference of pJ34 with transformation of pKFKOorf60), (2) growth of the transformants at permissive temperature in the presence of erythromycin, and (3) shift to non-permissive temperature of 40 °C for ca. 6 h, single-colony-isolates harbouring pKFKOorf60 integrated into *orf60*, resulting in knock-out of the gene, were isolated. For proving integration at the predicted site, genomic DNA was isolated from single-colony-isolates and PCR assays were performed using primer TP-J34orf60F (5'-CACCTGAACCAGAACC-3') binding in *orf60* of prophage TP-J34 and pGhEcoRI (5'-TTACACGTTACTAAAGGG-3') binding in pG$^+$host9, respectively. The size of the amplified PCR-product of ca. 630 bp corresponded well to the theoretical value of 627 bp (not shown).

## 3. Results

### Growth behaviour of *Streptococcus thermophilus* strains

The two *S. thermophilus* strains J34 and J34-6 had previously been shown in over-night liquid cultures to differ in their growth behaviour: while lysogenic strain J34 grew homogeneously, prophage-cured strain J34-6 showed a sedimenting phenotype (Neve *et al.*, 2003). As shown in Figure 1, the turbidity of *S. thermophilus* J34 batch cultures grown into stationary phase did not change within 30 h, while J34-6 cells sedimented rapidly after 5 h into stationary phase. While *S. thermophilus* strain J34 formed rather short chains, strain J34-6 predominantly formed long chains.

### *In vitro* adherence and hydrophobicity of *Streptococcus thermophilus* and human pathogens

The lysogenic *S. thermophilus* strain J34 revealed a higher adherence efficiency to HT29 enterocytes (34%) than its prophage-cured, sedimenting derivative J34-6 (26%) (Figure 2). Similar high *in vitro* adherence levels were observed for *S. aureus* 6732 (31%), whereas adherence of *S. enteritidis* S489 and *L. monocytogenes* Scott A cells was lower (9 and 18%, respectively) (Figure 2). The BATH assays (Doyle and Rosenberg, 1995) showed that the hydrophobicity of the cell surface of prophage-cured *S. thermophilus* strain J34-6 was higher (91%) than for the lysogenic wild-type strain J34 (79%). Cells of *L. monocytogenes* strain Scott A revealed the highest surface hydrophobicity value (95%), while those of the two remaining pathogenic strains were significantly lower (i.e. 5% for *S. aureus* 6732 and 18% for *S. enteritidis* S489, respectively) (Figure 2).

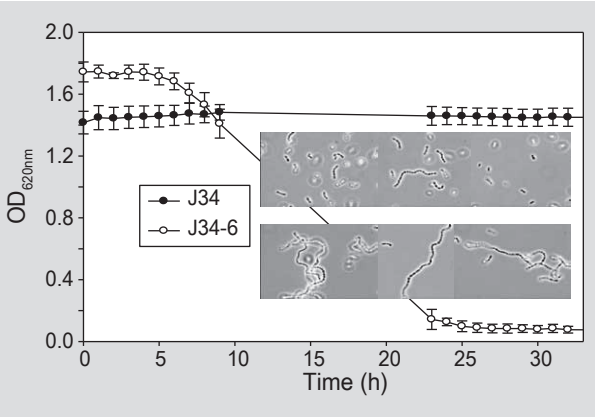


**Figure 1. Sedimentation behaviour of batch cultures of *Streptococcus thermophiles* strains J34 and J34-6. Cultures were grown to stationary phase, shaken for even distribution of cells, and then left standing at room temperature without further shaking. The half-tone inserts show representative phase-contrast microscopy pictures of J34 (upper) and J34-6 cells (lower) at early stationary phase (t=0).**

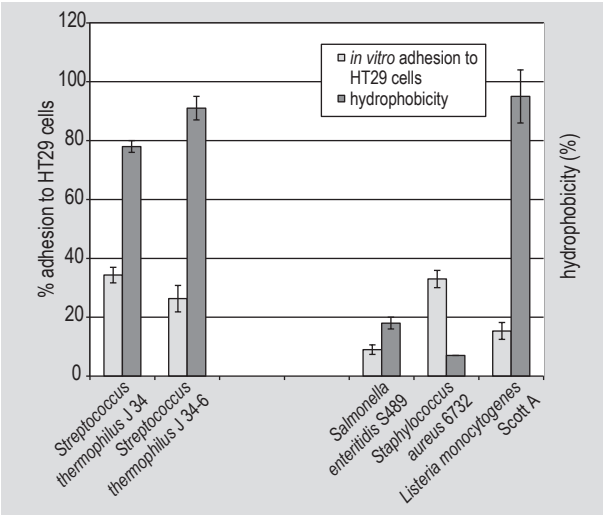


**Figure 2. *In vitro* adhesion and hydrophobicity of *Streptococcus thermophilus* J34 and J34-6 and three human pathogens. Values presented are mean ± standard deviation from three independent experiments (n=9).**

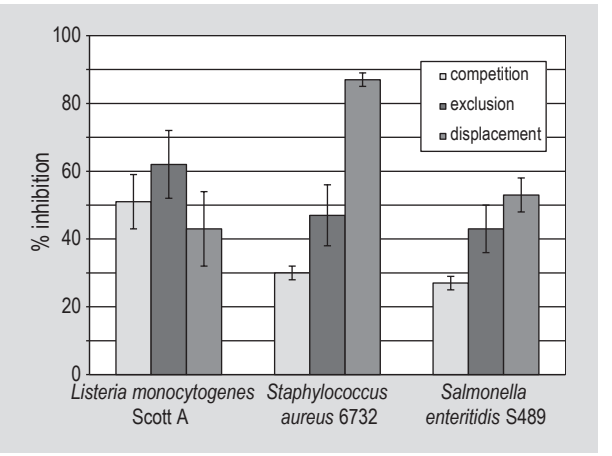


**Figure 3. Inhibition of *in vitro* pathogen adhesion by *Streptococcus thermophilus* J34. For competition assay, *S. thermophilus* cells were added to enterocytes simultaneously with the three pathogens. For exclusion assay, *S. thermophilus* cells were added 30 min before the addition of pathogens. For displacement assay, *S. thermophilus* cells LAB were added 30 min after addition of pathogens. Values presented are mean ± standard deviation from three independent experiments.**

### Interference of *Streptococcus thermophilus* with pathogens adhesion

In the *in vitro* exclusion assay with *L. monocytogenes* Scott A, the highest inhibition rate for strain *S. thermophilus* J34 was 63%, followed by an inhibition of 52% in the competition test. Only 45% of the adhered cells were subject to displacement. Adhesion of *S. aureus* 6732 was reduced by *S. thermophilus* J34 by only 30% when competition was measured. Values increased to 45% in the assay simulating exclusion and to even 85% when displacement was measured. Against *S. enteritidis* S489, the best inhibition result (53%) was seen in the assay simulating displacement. For competition and exclusion, inhibition was found to be 27 and 43%, respectively (Figure 3).

Using *S. thermophilus* J34-6, the highest inhibition rate (50%) was seen in the exclusion assay against *St. aureus* 6732. With an inhibition value of 30% against this pathogen, J34-6 was also effective in the assay simulating displacement. However, no inhibition was seen in the competition assay. Similar results were found in the tests with *L. monocytogenes* Scott A, the highest inhibition rate being 42% in the exclusion test. In the *in vitro* assay simulating displacement, 25% inhibition was observed, while this value dropped to 4% in the competition assay. Concerning inhibition of *S. enteritidis* S489 adhesion to HT29 cells by *S. thermophilus* J34-6, no inhibition at all could be seen regardless of the type of assay (Figure 4).

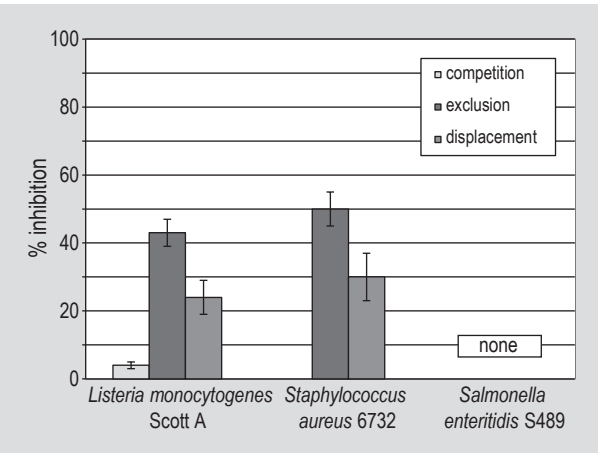


**Figure 4. Inhibition of *in vitro* pathogen adhesion by *Streptococcus thermophilus* J34-6. For competition assay, *S. thermophilus* cells were added to enterocytes simultaneously with the three pathogens, for exclusion assay, *S. thermophilus* cells were added 30 min before the addition of pathogens, for displacement assay, *S. thermophilus* cells were added 30 min after addition of pathogens. Values presented are mean ± standard deviation from three independent experiments (n=9).**

### Involvement of *orf60* of TP-J34 in growth of *Streptococcus thermophilus* J34

To identify genes involved in homogenous growth of lysogenic strain J34 we isolated random genome insertions of integrative plasmid pGhost9:IS*S1* (Maguin *et al.,* 1996) transformed into J34-12f. One insertion mutant, which exhibited sedimenting behaviour, was found to have

suffered an insertion in the genome of prophage TP-J34. By sequencing the DNA flanking the inserted plasmid on both sides, the place of insertion, identified by the duplicated target sequence CTTGACAG, was found at position 45382-45387 of the TP-J34 genome (Accession No. NC_020197; Ali *et al.,* 2014). This place was within *orf60*, a gene located in the region previously identified to encode genes involved in lysogenic conversion (Brüssow and Desiere, 2001). To verify that the knock-out of *orf60* was indeed the cause of aggregation, we cloned an internal part of *orf60* into pGhost9 and integrated the corresponding plasmid pKFKOorf60 by homologous recombination into the TP-J34 prophage genome in strain *S. thermophilus* J34f-2. Correct insertion was demonstrated by PCR applying primers binding to pGhost9 DNA and TP-J34 prophage DNA outside of the cloned internal *orf60* fragment, respectively. This knock-out of *orf60* caused an aggregating phenotype of the corresponding lysogenic host *S. thermophilus* J34f-2-KOorf60 (Figure 5).

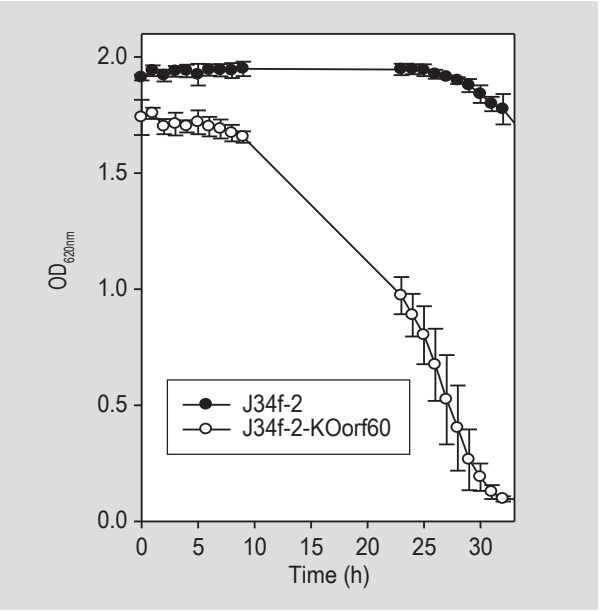


**Figure 5. Effect of *orf60* knock out on aggregation of *Streptococcus thermophilus* J34f-2-KOorf60. Cultures were grown to stationary phase, shaken for even distribution of cells, and then left standing at room temperature without further shaking. Means ± standard deviation of three independent experiments are shown.**

## 4. Discussion and conclusion

The ability to establish in or attach to the epithelium of the gastrointestinal tract is one of the most important prerequisites for microorganisms supposed to exert beneficial effects in the gastrointestinal tract (Bernet *et al.*, 1994; Fernandes *et al.,* 2003). Concerning the mechanisms underlying probiotic effects, competitive exclusion of potential pathogens is besides colonization resistance a widespread one among probiotics (Hill *et al.,* 2014).

When incubated together, *S. thermophilus* J34 but not *S. thermophilus* J34-6 was able to compete with three gastrointestinal pathogens for adhesion sites on the surface of HT29 cells. In particular, strain *S. thermophilus* J34 was more effective against *L. monocytogenes* Scott A. Once adhered, pathogens were excluded by *S. thermophilus* J34 by different degrees; the profile of pathogen exclusion was similar to that of competition. In the case of *S. thermophilus* J34, pathogen inhibition by displacement against the *S. aureus* and *S. enteritidis* strains were higher than the degree of inhibition achieved by competition and exclusion. In contrast to these results, *S. thermophilus* J34-6 was less effective in inhibiting colonisation of HT29 enterocytes by the three pathogens tested. Towards *S. enteritidis* S489, no inhibition at all of *in vitro* adhesion could be seen.

It is widely accepted that commensal or probiotic organisms may prevent colonization of enteropathogens on the gut epithelial surface by competitive exclusion (Banerjee *et al.*, 2009). Potential protective mechanisms against pathogens include adhesion to and colonization of the mucosal surfaces through competition for binding sites and nutrients (Collado *et al.*, 2007b). Sharing of common carbohydrate-binding sites in probiotic organisms allows the blocking of adhesion receptors, thus promoting the inhibition of pathogen adhesion by steric hindrance (Gueimonde *et al.*, 2006; Lee *et al.*, 2003). Apart from whole cell binding, soluble factors may also be involved (which may cause the inhibition of adhesion or colonisation of enteropathogens, e.g. loosely adhered surface proteins of certain lactobacilli released in the gut lumen (Gueimonde *et al.*, 2006; Sun *et al.*, 2007). In studies with *L. delbrueckii* subsp. *bulgaricus,* Banerjee *et al.* (2009) hypothesized that bioactive components released by *L. delbrueckii* subsp. *bulgaricus* are causing inhibition of pathogen adhesion. Possible mechanisms involved may be proteolytic cleavage of toxin or toxin receptors, blockage of toxin receptors or blockage of pathogen adhesion molecules on host cells by competitive binding by the bioactive agents. In addition, probiotic *S. thermophilus* and *Lactobacillus acidophilus* strains reduced adhesion and invasion of enteroinvasive *Escherichia coli* cells and increased transepithelial resistance and tight junction integrity during infection (Resta-Lennert *et al.*, 2003). Exposure of epithelial cells to *Lactobacillus casei* prior to infection with adherent-invasive *E. coli* reduced adhesion of the pathogen by 75% (Ingrassia *et al.*, 2005). Physiological stressors may alter susceptibility of the host epithelium to infection by enteric pathogens. Studies of Burkholder *et al.* (2009) revealed that adhesion of *Salmonella* to Caco-2 cell during thermal stress (41 °C, 2 h) was significantly reduced by *Lactobacillus rhamnosus* GG in pre-exposure assays. Studying *Lactobacillus* and *Bifidobacterium* strains, Candela *et al.* (2008) found these strains being effective in displacing enteropathogens

from a Caco-2 cell layer. When the same strains were assessed for their immunomodulatory activity on IL-8 production by HT29 cells, they showed the potential to protect enterocytes from an acute inflammatory response. In order to manifest beneficial effects, probiotic bacteria need to achieve an adequate mass through aggregation. Organisms with the ability to co-aggregate with pathogens may have significant advantages over non co-aggregating organisms which are easily removed from the intestinal environment (Collado *et al.*, 2007a). It has been shown that the co-aggregation abilities of probiotic strains might enable them to form a barrier that prevents colonization by pathogenic bacteria (Collado *et al.*, 2007a; Schachtsieck *et al.*, 2004; Schellenberg *et al.*, 2006) This is different from the results of this present study, where aggregation was apparently caused by chain formation of the cells and less so by altered surface properties. It appears that the latter properties are important for co-aggregation as described previously (Schachtsieck *et al.*, 2004; Schellenberg *et al.*, 2006; Collado *et al.*, 2007a).

The prophage gene *orf60*, identified to be involved in homogeneous growth of *S. thermophilus* J34, encodes an apparently cytoplasmic protein, which seems to be associated with temperate phages in the genus *Streptococcus*, where it is encoded in the region of lysogenic conversion, located downstream of the lysis module (Brüssow and Desiere, 2001). BlastP analyses (Altschul *et al.*, 1997) showed that genes encoding similar proteins are found in several of the temperate phages of *S. thermophilus*: 5093, 20617, TP-778L, Sfi21, and O1205, with E-values ranging from 1e-79 to 6e-64. To none of these proteins a function has been assigned. Since *orf60* encodes an apparently cytoplasmic protein, it is tempting to speculate that it could be a regulatory protein affecting the chain-forming properties of the host cells by either influencing surface structural components or cell division. Certainly, more work is needed to clarify the function of the *orf60* gene product.

Despite of the intensive aggregation of *S. thermophilus* J34-6 (compared to *S. thermophilus* J34, see introduction), this strain was found to be less effective than strain J34 in reducing *L. monocytogenes* Scott A, *S. aureus* 6732 and *S. enteritidis* S489 adhesion to enterocytes in the HT29 model. Although the specific mechanisms of diminished adhesion to the three pathogens tested remains to be elucidated, results of this study indicate a potential protective role of *S. thermophilus* J34 against infections caused by these three pathogenic strains. Thus, the prophage residing in *S. thermophilus* J34 may confer a potentially beneficial effect on the human host through its host bacterium. This is quite surprising, since many prophages are known to confer adverse properties to the human host through their host bacteria (Nanda *et al.*, 2015; Tinsley *et al.*, 2006). An example, which has been known for more than 50 years, is the lysogenic *Corynebacterium diphtheriae* harbouring a prophage encoding the diphtheria toxin (Freeman, 1951). During recent years, beneficial effects of prophages on their host bacteria have been described, which provide increased fitness to their bacterial hosts (Canchaya *et al.*, 2004; Nanda *et al.*, 2015). However, if fitness of a beneficial bacterium is increased, this should also be beneficial for the human or animal hosts, who take advantage of the beneficial effects of the bacterium. The effects described in this study appear to differ from this general view in that the beneficial effect described may be of potential importance for the human or animal host and less so for the bacterial host.

*S. thermophilus* is known for its low resistance to the conditions of the upper gastrointestinal tract (Conway *et al.*, 1987; Lick *et al.*, 2001). This may prevent its unprotected application as a probiotic in food, except for its application in yoghurt for lactose digestion in lactose malabsorbers (EFSA, 2010), where feeding to minipigs of bacteria killed under conditions not inactivating β-galactosidase was shown to be equally effective as feeding live bacteria (de Vrese *et al.*, 2001). However, considering the progress realised in encapsulation techniques in recent years (De Vos *et al.*, 2010), application of *S. thermophilus* for achieving probiotic effects in the lower gastrointestinal tract should be possible. By encapsulation, another problem may be circumvented, which we have not yet addressed. We have grown our cells in a transparent defined medium for the obvious reason that aggregation could be easily monitored. Since *S. thermophilus* is predominantly applied in yoghurt fermentation, we should control whether *S. thermophilus* J34 maintains its properties of homogeneous growth when grown in milk alone or in combination with *L. delbrueckii* subsp. *bulgaricus*.

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