**Isolated phages and their use as disinfectant in food or for sanitation of factory environment.**

**Field of the invention**

The present invention relates to phage isolates having a strong lytic activity against *Enterobacter sakazakii* strains and their use as anti-microbial agent in food products, in particular infant formula, and for sanitation of factory environments.

**Background of the invention**

Enterobacteria represent a dominant portion of the "recontamination flora" of dairy products made from pasteurised milk. Their presence often indicates inadequate sanitization and/or faulty construction of the processing equipment. Members of the genus *Enterobacter* are frequently found in dried products such as milk powder.

Among the *Enterobacteria, E. sakazakii* can cause serious infections especially among very young. It has been implicated in a rare but severe form of neonatal meningitis among neonates and infants, with dried-infant formula being implicated as the mode of transmission.

Aseptic manufacturing of dried infant formula is not feasible because the costs for the production will be too elevated. Therefore, the manufacturers rely on the use of disinfectants and specific hygienic work zones during processing to provide safe products. Nevertheless, low numbers of bacteria can grow to considerable counts when a reconstituted infant formula is not consumed rapidly and kept at ambient temperature. This may render the product potentially dangerous for the infant consuming it.

In view of the deficiencies in the prior art, the problem of the present invention resides in providing a food, in particular milk based products such as infant formulas, which may be safe even after storage in conditions favouring the growth of *E.*

*sakazakii.* Also, the invention aims to develop a safe agent that can be used for decontamination of *E. sakazakii* in any food product and for sanitation of factory environments.

This problem has been solved by providing bacteriophage preparations with substantial lytic potential towards *E. sakazakii*, that are safe and non-toxic.

**Summary of the invention**

It is an object of the present invention to develop new and useful phage isolates with substantial lytic potential towards *E. sakazakii* and which can be used alone or in cocktail to prevent or to treat food products and factory environments from contaminations caused by *E. sakazakii*, in particular infant formulas.

It is thus another object of the present invention to provide a food product that contains phage isolates selected to lyse E.sakazakii., in particular a milk based formula, a nutritional composition, a pet food product, a dietary supplement or any food at risk of contamination by *E. sakazakii*.

It is a further object of the present invention to provide an agent for decontamination of food product and sanitation of factory environment, which comprises an effective amount of at least one phage isolate or cocktail as described above.

In another embodiment the present invention relates to the use of at least one phage isolate or cocktail as described above, for decontamination of food products and sanitation of factory environment or in the preparation of a composition intended to prevent or treat infections caused by *E. sakazakii* in humans or animals.

It is yet another object of the present invention to provide a method of decontamination of food products or sanitation of factory environment, which comprises using at least one bacteriophage preparation with substantial lytic potential towards *E. sakazaki* as described above.

It further relates to a method for selecting phage isolate with substantial lytic potential towards *E. sakazakii* as described above.

A major advantage of the present invention is that it provides for a decontaminant having a high specificity for *E. sakazakii*. It may prevent contamination and outgrowth of *E. sakazakii* in any food product, being particularly useful for milk based products such as infant formulas. It can also be efficient for the treatment or prevention of infections caused by *E. sakazakii* in humans or animals.

Another advantage of the present invention is that it provides an agent which helps to disinfect, clean and decontaminate working surfaces or processing equipment in factories, as well as floors; walls and the like.

Another advantage of the present invention is that it provides an agent that is environmentally safe and non-toxic for humans.

**Detailed Description of the Invention**

Within the following description, the term "FSM-phage" designates the Food Safety Microbiology bacteriophage collection (Nestlé Research Centre, Vers-chez-les-Blanc, Lausanne, Switzerland).

According to a first aspect, an agent for decontamination of food and sanitation of factory environment, comprises at least one phage isolate with substantial lytic potential towards *E. sakazakii*, is concerned.

The phages according to the present invention may be obtained by screening candidate bacteriophages for those which infect *E. sakazakii* strains using the spot assay test (Pelczar, M.J., E.C.S. Chan, and N.R. Krieg. Microbiology concepts and applications, Chapter 16 Viruses: cultivation methods, pathogencity p.436-452, McGraw-Hill, Inc. New York 1993), or in liquid culture (H.W. Ackermann and M.S.

DuBow. Viruses of Prokaryotes volume 1. Chapter 6. Description and identification of new phages p. 103-142. CRC Press Inc, Boca Raton, Florida, USA.). The method for selecting the Bacteriophages according to the present invention is detailled in example 1. Preferably, the phage isolate having the ability to infect :

i) at least 9 bacterial strains of *E.sakazakii* from the group consisting of FSM-16; FSM-33; FSM-261; FSM-265; FSM-266, FSM-269; FSM-270; FSM-271; FSM-272; FSM-273; FSM-274; FSM-280; FSM-281; FSM-284; FSM-286; FSM-288; FSM-290; FSM-292; FSM-297; FSM-298; FSM-300; FSM-303; FSM-305; FSM-308; FSM-309; FSM-311; FSM-313; FSM-314; FSM-316; FSM-318; FSM-322; FSM-323; FSM-1360; FSM-1387-2NL; FSM-MC7; FSM-MC8; FSM-MC9; FSM-MM10 and FSM-MM11, on solid medium and,

ii) at least 9 bacterial strains of *E.sakazakii* from the group consisting of FSM-16; FSM-33; FSM-261; FSM-265; FSM-266, FSM-269; FSM-270; FSM-271; FSM-272; FSM-273; FSM-274; FSM-280; FSM-281; FSM-284; FSM-286; FSM-288; FSM-290; FSM-292; FSM-297; FSM-298; FSM-300; FSM-303; FSM-305; FSM-308; FSM-309; FSM-311; FSM-313; FSM-314; FSM-316; FSM-318; FSM-322; FSM-323; FSM-1360; FSM-1387-2NL; FSM-MC7; FSM-MC8; FSM-MC9; FSM-MM10 and FSM-MM11, in liquid medium,

were selected.

In a preferred embodiment, the phage isolates are FSM-Phage 67/33/1, FSM-Phage F, FSM-Phage 9/261, FSM-Phage F/316and FSM-Phage 73/311/2, FSM-Phage 73/261, FSM-Phage 33/33/1, FSM-Phage 28/145/2 and FSM-Phage 10/261/1.

In a most preferred embodiment, the phages FSM-Phage 10/261/1, FSM-Phage 73/261, FSM-Phage 33/33/1, FSM-Phage 67/33/1, FSM-Phage F/316, FSM-Phage 9/261, and FSM-Phage 73/311/2 together with their corresponding propagation strains have been deposited by way of example at the Institut Pasteur, 28 rue du Docteur Roux, F-75024 Paris CEDEX 15, FRANCE, on 14/11/2003, under the deposit number CNCM I-3127, CNCM I-3128, CNCM I-3129, CNCM I-3130, CNCM I- 3131, CNCM I-3132 and CNCM I-3133, respectively.

Advantageously, the phages deposited are able to lyse at least 80 % out of the *E. sakazakii* from the Nestlé Research Centre collection composed of 39 strains. This collection is representative for the *E. sakazakii* that can be found in food products, in particular infant formula. With the phage material according to the invention, coverage of about 97 per cent can be obtained by using a phage cocktail of at least two phages. Preferably, the phages are not able to recognize non-related bacteria such as the gram-negative lactic acid bacteria.

Thus, according to a further aspect, the invention relates to the use of at least one phage isolate as described above, in any food product or nutritional supplement, for preventing contamination and outgrowth of *E. sakazakii*. Examples for food or pharmaceuticals products are milk, yoghurt, curd, cheese, fermented milks, milk based fermented products, ice-creams, fermented cereal based products, milk based powders, infant formulae or tablets, liquid suspensions, dried oral supplement, wet oral supplement, dry-tube-feeding. Methods for preparing them are common knowledge.

The composition according to the invention may also comprise usual excipients, in particular sweeteners, flavouring agents or preservatives. It can further comprise a prebiotic and/or a probiotic microorganism. The compositions of the invention may be formulated according to any one of a number of techniques that are well known to this art.

In another embodiment, a food composition containing at least one phage isolate according to the present invention is prepared. This composition may be a nutritional complete formula, an infant formula, a dairy product, a chilled or shelf stable beverage, water, a soup, a dietary supplement, a meal replacement, a nutritional bar or a confectionery. In a most preferred embodiment, phages may be added to a dry powdered infant formula.

In another embodiment, a usual food product may be enriched with at least one phage isolate according to the present invention. For example, a fermented milk, a yoghurt, a fresh cheese, a renneted milk, article of confectionery, for example a sweet or sweetened beverage, a confectionery bar, breakfast cereal flakes or bars, drinks, milk powders, soy-based products, non-milk fermented products or nutritional supplements for clinical nutrition.

Once a phage isolate has been selected according to the present invention, said phage isolate may be included alone or in a phage cocktail, into a product as mentioned above in an amount of at least about $10^4$ pfu (plaque forming unit)/ml or gram (depending if the phages are added in a dry or liquid state), and more preferably from about $10^6$ to about $10^{11}$ pfu/ml or gram. The phage isolate may be prepared by spray-drying, freeze-drying, or in liquid sample container using the product as support material, e.g. milk powder for infant formula. It can also be used as a supplement to be mixed with the product before serving, as additional packaging (e.g. sachet).

Also, application of phages according to the invention or spraying or otherwise applying phages in a factory environment is performed with the objective to inhibit growth of the target bacteria. Such compositions may be prepared by conventional methods. For this application, the amount of phages is preferably of at least about $10^6$ pfu (plaque forming unit)/ml or gram, and more preferably from about $10^6$ to about $10^{11}$ pfu/ml or gram.

The following examples are given by way of illustration only and in no way should be construed as limiting the subject matter of the present application. The examples are preceded by a brief description of the figures.

**Figures 1** is a diagram showing the growth of the *E. sakazakii* strain mixture (FSMCC # 145, 286, 290, 305, 1987/2NL) in BHI broth inoculated with $10^8$ pfu/ml phage cocktail (FSMCC-phage # 67/33/1, F/316, 9/261, 73/311/2) (●) or without phage cocktail (▲).

**Figure 2** is a diagram showing the growth of the *E. sakazakii* strain mixture (FSMCC # 145, 286, 290, 305, 1987/2NL) in Reconstituted Infant Formulas inoculated with $10^8$ pfu/ml phage cocktail (FSMCC-phage # 67/33/1, F/316, 9/261, 73/311/2) (●) or without phage cocktail (▲).

**Figure 3** is a diagram showing the growth of the *E. sakazakii* strain mixture (FSMCC # 145, 286, 290, 305, 1987/2NL) on the surface of stainless steel discs treated with $10^8$ pfu/ml phage in the phage cocktail (FSMCC-phage # 67/33/1, F/316, 9/261, 73/311/2) (●), or without phage cocktail (▲).

**Examples**

**Example 1 : Selection of phages according to the invention**

A collection of 114 phage isolates originating from the Nestlé Food Safety Microbiology bacteriophage collection, were tested against 39 strains of *E. sakazakii* originating from Food Safety Microbiology culture collection (FSMCC). The strains are listed in Table 1 and 2, respectively.

| | Origin | FSMCC n° | | Origin | FSMCC n° |
|---|---|---|---|---|---|
| 1 | factory environment | 9/145/1 | 58 | Sewage (primary mud) | 64/33/2 |
| 2 | " | 9/145/2 | 59 | " | 64/311 |
| 3 | " | 9/145/3 | 60 | Sewage (channel rejection in the lake) | 66/311/1 |
| 4 | " | 9/145/4 | 61 | " | 66/311/2 |
| 5 | " | 9/145/5 | 62 | " | 67/33/1 |
| 6 | " | 9/261 | 63 | " | 67/33/2 |
| 7 | " | 9/261/2 | 64 | " | 67/300/1 |
| 8 | " | 10/145/1 | 65 | " | 67/300/2 |
| 9 | " | 10/145/2 | 66 | " | 67/311/1 |
| 10 | " | 10/145/3 | 67 | " | 67/311/2 |
| 11 | " | 10/145/4 | 68 | Sewage (arrival of water before filtration on sand) | 61/MC9/1 |
| 12 | " | 10/261/1 | 69 | " | 61/MC9/2 |
| 13 | " | 10/261/2 | 70 | " | 61/MC9/3 |
| 14 | " | 10/261/3 | 71 | Sewage (primary mud) | 64/MC9/1 |
| 15 | " | 10/261/4 | 72 | " | 64/MC9/2 |
| 16 | " | 10/261/5 | 73 | Sewage (channel rejection in | 66/MC9/1 |

| | | | | | |
|---|---|---|---|---|---|
| | | | | the lake) | |
| 17 | Waste water | 16/145 | 74 | " | 66/MC9/2 |
| 18 | Factory environment | 22/145/1 | 75 | " | 67/MC9/1 |
| 19 | " | 22/145/2 | 76 | " | 67/MC9/2 |
| 20 | " | 22/145/3 | 77 | Sewage (entry of water) | 73/33/1 |
| 21 | " | 22/145/4 | 78 | " | 73/33/2 |
| 22 | " | 22/145/4 | 79 | " | 73/261/1 |
| 23 | " | 23/16 | 80 | " | 73/261/2 |
| 24 | " | 23/145/1 | 81 | " | 73/300/1 |
| 25 | " | 23/145/2 | 82 | " | 73/300/2 |
| 26 | " | 23/145/3 | 83 | " | 73/311/1 |
| 27 | " | 23/145/4 | 84 | " | 73/311/2 |
| 28 | " | 23/145/5 | 85 | " | 73/MC9/1 |
| 29 | " | 23/1387 | 86 | " | 73/MC9/2 |
| 30 | " | 28/16 | 87 | Sewage (exit of water) | 74/MC9/1 |
| 31 | " | 28/145/1 | 88 | " | 74/MC9/2 |
| 32 | " | 28/145/2 | 89 | Sewage (biological water) | 75/311/1 |
| 33 | " | 33/33/1 | 90 | " | 75/311/2 |
| 34 | " | 33/33/2 | 91 | " | 75/MC9/1 |
| 35 | " | 33/33/3 | 92 | " | 75/MC9/2 |
| 36 | " | 33/33/4 | 93 | Sewage (entry of water) | 76/33/1 |
| 37 | " | 33/33/5 | 94 | " | 76/33/2 |
| 38 | " | 46/MC9/1 | 95 | " | 76/311/1 |
| 39 | " | 46/MC9/2 | 96 | " | 76/311/2 |
| 40 | " | 58/MC9/1 | 97 | " | 76/311/3 |
| 41 | " | 58/MC9/2 | 98 | " | 76/311/4 |
| 42 | " | 58/MC9/3 | 99 | " | 76/311/5 |
| 43 | Sewage | 61/33/5 | 100 | " | 76/MC9/1 |
| 44 | " | 61/33/6 | 101 | " | 76/MC9/2 |
| 45 | " | 61/33/7 | 102 | Sewage (exit of water) | 77/33/1 |
| 46 | " | 61/33/8 | 103 | " | 77/33/2 |
| 47 | " | 61/300 | 104 | " | 77/311/1 |
| 48 | " | 61/300/1 | 105 | " | 77/311/2 |
| 49 | " | 61/300/2 | 106 | " | 77/MC9/1 |
| 50 | " | 61/311/1 | 107 | " | 77/MC9/2 |
| 51 | " | 61/311/2 | 108 | Sewage (biological water) | 78/33/1 |
| 52 | " | 61/311/3 | 109 | " | 78/33/2 |
| 53 | " | 61/311/4 | 110 | " | 78/311/1 |
| 54 | " | 61/311/5 | 111 | " | 78/311/2 |
| 55 | " | 62/300/1 | 112 | " | 78/MC9/1 |
| 56 | " | 62/300/2 | 113 | " | 78/MC9/2 |
| 57 | Sewage (primary mud) | 64/33/1 | 114 | Factory environment | F/316 |

**Table 1: Phage isolates investigated**

| FSM-Code | Origin | FSM-Code | Origin |
|---|---|---|---|
| Mal-16 (16) | Finished product: cereals | 300 | Milk Factory environment |
| 33 | Infant formula | 303 | Milk Factory environment |
| 261 | Munich University | 305 | Milk Factory environment |
| 265 | Unknown | 308 | Milk Factory environment |
| 266 | Unknown | 309 | Milk Factory environment |
| 269 | Milk Factory environment | 311 | Milk Factory environment |
| 270 | Milk Factory environment | 313 | Milk Factory environment |
| 271 | Milk Factory environment | 314 | Milk Factory environment |
| 272 | Milk Factory environment | 316 | Milk Factory environment |
| 273 | Milk Factory environment | 318 | Milk Factory environment |
| 274 | Milk Factory environment | 322 | Milk Factory environment |
| 280 | Milk Factory environment | 323 | Milk Factory environment |
| 281 | Milk Factory environment | 1360 | Milk Factory environment |
| 284 | Milk Factory environment | 1387-2NL | Milk Factory environment |
| 286 | Milk Factory environment | MC7 | Wageningen University |
| 288 | Milk Factory environment | MC8 | Wageningen University |
| 290 | Milk Factory environment | MC9 | Wageningen University |
| 292 | Milk Factory environment | MM10 | Wageningen University |
| 297 | Milk Factory environment | MM11 | Wageningen University |
| 298 | Milk Factory environment | | |

**Table 2: *Enterobacter sakazakii* strains**

## SCREENING ON SOLID MEDIUM

*Isolation of plaques and phage amplification*

Sampling:

Take sewage water samples (approx. 50 ml) for the research for bacteriophages. Measure the pH of each sample using pH paper strips (if pH lower than 5, to adjust to 7 with NaOH 1N).

The samples are then submitted to the following steps:

1. Centrifugation 10 min at 2500g (to eliminate the large remains).
2. Filtration of the supernatant using a filter of 0.20 µm (to eliminate bacteria)
3. Perform the tests as follows or keep samples at 4°C for 1 or 2 days MAXIMUM.

For the isolation of bacteriophages, the above pre-treated samples have been tested in parallel with or without pre-amplification. Once the presence of phage is detected, they are amplified and stored.

**Screening without pre-amplification:**

*Step 1: Preparation of the bacterial culture:*

A culture of *E. sakazakii* having grown overnight at 30°C in Brain Heart Infusion (BHI, Oxoid CM225) is prepared, and then 0.5 ml of this culture is transferred in 4ml of fresh BHI and incubated 1h at 40°C.

*Step 2: Add samples to bacteria:*

Add in a tube containing 3ml soft BHI agar (BHI broth + 0.6% agar) maintained at 45 ± 1°C, 100 µl of the *E. sakazakii* culture (prepared as above) + 100 µl of treated sample to be tested.

*Step 3: Inoculation of Petri dishes:*

Mix the tube content with a Vortex (± 2sec.). Then, pour the whole tube content on the surface of a Petri dish containing a thin layer of BHI agar and let solidify on the bench. Incubate inverted plates overnight at 30°C.

*Step 4: Interpretation of results:*

Note the presence or absence of plaques of lysis on each Petri dish.

*Step 5: Controls*

Prepare a positive control (*E. sakazakii* strain n° 261 + active phage against this strain at a concentration of 2-3 log pfu/ml): Apply steps 1 to 4.

Prepare a negative control without phage added: Apply steps 1 to 4 without addition of phage or sample to be tested.

**Screening with pre-amplification:**

*Step 1 – Day 1: Preparation of the bacterial culture:*

A culture of *E. sakazakii* having grown overnight at 30°C in Brain Heart Infusion (BHI, Oxoid CM225) is prepared, then 0.5 ml of this culture is transferred in 4ml of fresh BHI and incubated 1h at 40°C.

*Step 2 – Day 1: Amplification of phage in the sample:*

In a tube containing 4ml BHI broth, 100µl of the *E. sakazakii* culture (prepared as in step 1) + 1 ml of treated sample to be tested, are added and incubated 3-5h at 30°C under agitation at 150-200 rpm. They are left overnight on the bench.

*Step 3 – Day 2:* Centrifugation 10 min at 2500 rpm

*Step 4 – Day 2: Preparation of the bacterial culture:*

A culture of *E. sakazakii* having grown overnight at 30°C in Brain Heart Infusion (BHI, Oxoid CM225) is prepared, then 0.5 ml of this culture is transferred in 4ml of fresh BHI and incubated 1h at 40°C.

*Step 5 – Day 2: Add samples to bacteria:*

Add in a tube containing 3ml soft BHI agar (BHI broth + 0.6% agar) maintained at 45 ± 1°C, 100 µl of the *E. sakazakii* culture (prepared as above) + 100 µl of sample obtained in step 3.

*Step 6 – Day 2: Inoculation of Petri dishes:*

Mix the tube content with a Vortex (± 2sec.). Then, pour the whole tube content on the surface of a Petri dish containing a thin layer of BHI agar and let solidify on the bench. Incubate inverted plates overnight at 30°C.

*Step 7 – Day 3: Interpretation of results:* Note the presence or absence of plaques of lyses on each Petri dish.

When the presence of phage is detected, they are amplified and stored.

**Amplification of a lyses plaque**

*Step 1: Preparation of the bacterial culture:*

A culture of *E. sakazakii* having grown overnight at 30°C in Brain Heart Infusion (BHI, Oxoid CM225) is prepared, then 0.5 ml of this culture is transferred in 4ml of fresh BHI and incubated 1h at 40°C.

*Step 2: Amplification of the pfu:*

In a tube containing 4ml BHI broth, 100 µl of the *E. sakazakii* culture (prepared as in step 1) + 1 isolated pfu (cut from Agar) are added and incubated 3-5h at 30°C under agitation at 150-200 rpm. Tubes are left overnight on the bench.

*Step 3:* Centrifugation 10 min at 2500rpm.

*Step 4:* Filtration of the supernatant using a filter of 0.20 µm (to eliminate bacteria)

***Enumeration of phages***

*Step 5: Preparation of the bacterial culture:*

A culture of *E. sakazakii* having grown overnight at 30°C in Brain Heart Infusion (BHI, Oxoid CM225) is prepared and then 0.5 ml of this culture is transferred in 4ml of fresh BHI and incubated 1h at 40°C.

*Step 6: Add phages to bacteria:*

Add in a tube containing 3ml soft BHI agar (BHI broth + 0.6% agar) maintained at 45 ± 1°C, 100 µl of the *E. sakazakii* culture (prepared as above) + 100 µl of serially diluted sample obtained in step 4.

*Step 7: Inoculation of Petri dishes:*

Mix the tube content with a Vortex (± 2sec.) and pour the whole tube content on the surface of a Petri dish containing a thin layer of BHI agar. Then, let solidify on the bench and incubate inverted plates overnight at 30°C.

*Step 8: Interpretation of results:*
Enumerate lyses plaques on each Petri dish.

**Storage after amplification**

*Step 1: Preparation of aliquots:*
Distribute into cryo-tubes approx. 1.5ml of the filtered amplified phage solution (obtained in step 4 of the "Amplification of one lyses plaque" paragraph)

*Step 2: Addition of glycerol:*
In each tube 15% of sterile glycerol is added and mixed quickly using a Vortex.

*Step 3:* Quickly freeze the tubes in liquid nitrogen

*Step 4:* Storage of the tubes in a freezer at –20°C

**SCREENING IN LIQUID MEDIUM**

*Step 1, Day 0: Preparation of the bacterial culture:*
A culture of *E. sakazakii* having grown overnight at 30°C in Brain Heart Infusion (BHI, Oxoid CM225) is prepared.

*Step 2, Day 1: Preparation of the bacterial culture:*
Transfer 0.1 ml of this culture in 4ml of fresh BHI and incubated 6h at 40°C (stationary growth phase of cells). Dilute cells up to 3log cfu/ml in Tryptone Salt (TS, Oxoid L42).

*Step 3, Day 1: Microtiterplate assay*

Effectiveness of phages against *E. sakazakii* was investigated in a microtiterplate assay. Each plate was set up with each of the test microorganisms as follows: external wells, 200 µl of sterile BHI (blank control), other wells, 20 µl of diluted *E. sakazakii* prepared in step 2. Store overnight microtiterplates at 4°C.

*Step 4, Day 2: Microtiterplate assay*

Leave the microtiterplates from the refrigerator. Add in negative control wells, 180µl BHI and in test wells, 160µl BHI plus 20µl phage suspension (final concentration of 8 log pfu/ml). Read at 620nm the optical density (time zero) of the microtiterplate by using a microplate reader Multiskan MCC340. Plates are then incubated at 37°C in ambient air and $OD_{620nm}$ was read after 6h30 and 8h30 incubation.

*Step 5, Day 3: Microtiterplate assay*

After the last reading, plates are left at ambient temperature for one total duration of 24h. After which the $OD_{620nm}$ is last once read.

**RESULTS**

Following to the test on solid medium, phage isolates that are active against at least 9 out of the 39 *E. sakazakii* strains selected from the group :FSM-16; 33; 261; 265; 266, 269; 270; 271; 272; 273; 274; 280; 281; 284; 286; 288; 290; 292; 297; 298; 300; 303; 305; 308; 309; 311; 313; 314; 316; 318; 322; 323; 1360; 1387/2NL; MC7; MC8; MC9; MM10 and MM11, are selected. Thus, the 11 phage isolates (including the phages FSM-Phage 67/33/1, FSM-Phage F/316, FSM-Phage 9/261, and FSM-Phage 73/311/2) are presented in Table 3. Some phage isolates proved to be efficient against at least 20 out of the 39 bacterial strains.

| *Enterobacter sakazakii* strain number | Bacteriophage number (FSM-phage) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | F/316 | 73/26 1/1 | 9/261 | 28/14 5/2 | 23/16 | 10/26 1/1 | 22/14 5/3 | 28/16 | 33/33 /1 | 67/33 /1 | 73/31 1/2 |
| 16 | 2 | 2 | 2 | 2 | 2 | | 2 | 2 | 0 | 0 | 0 |
| 33 | 2 | 0 | 2 | 2 | 0 | 0 | 0 | 0 | 2 | 2 | 2 |
| 261 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 0 |
| 265 | 2 | 2 | 2 | | | | | | 2 | 0 | 0 |
| 266 | 2 | 2 | 2 | | | | | | 2 | 2 | 0 |
| 269 | 2 | 2 | 2 | | | | | | 0 | 0 | 0 |
| 270 | 2 | 0 | 2 | | | | | | 0 | 2 | 0 |
| 271 | 2 | 2 | 2 | | | | | | 2 | 0 | 0 |
| 272 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 0 |
| 273 | 2 | 2 | 2 | | | | | | 2 | 0 | 0 |
| 274 | 2 | 2 | 2 | 0 | 0 | 2 | 0 | 0 | 0 | 2 | 2 |
| 280 | 2 | 2 | 2 | | | | | | 2 | 0 | 0 |
| 281 | 2 | 2 | 2 | | | | | | 2 | 0 | 0 |
| 284 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 0 |
| 286 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 0 | 0 |
| 288 | 2 | 2 | 2 | | | | | | 0 | 0 | 0 |
| 290 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 0 | 0 |
| 292 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 0 |
| 297 | 2 | 2 | 2 | | | | | | 0 | 0 | 0 |
| 298 | | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 |
| 300 | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 2 |
| 303 | 2 | 2 | 2 | 2 | 2 | 0 | 2 | 2 | 2 | 0 | 0 |
| 305 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 0 | 0 | 0 |
| 308 | | 2 | ? | | | | | | | 2 | 2 |
| 309 | | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 2 |
| 311 | 2 | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 2 |
| 313 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 0 | 0 |
| 314 | 2 | 2 | 2 | | | | | | 2 | 2 | 0 |
| 316 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 0 |
| 318 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 0 |
| 322 | 2 | 2 | 2 | | | | | | 2 | 2 | 0 |
| 323 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 0 |
| 1360 | 2 | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 |
| 1387/2NL | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 0 | 0 | 2 |
| MC7 | 0 | | 0 | 2 | 2 | 0 | | | | 0 | 0 |
| MC8 | 2 | | 2 | 2 | 2 | 2 | | | | 0 | 0 |
| MC9 | 2 | | 2 | 2 | 2 | 2 | | | | 2 | 2 |
| MM10 | 2 | | 2 | 2 | 2 | 2 | | | | 0 | 0 |
| MM11 | 2 | | 0 | 2 | 2 | 2 | | | | 2 | 0 |

**Table 3: Selection of the phages according to the results obtained in solid medium.**

**legend: 0 non active; 2 active.**

The said phages were further tested on liquid medium in order to select the phage isolates according to the present invention. The results are then presented in Table 4.

| | | Bacteriophage number (FSM-phage) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 67/33/1 | 10/261/1 | F/316 | 9/261 | 33/33/1 | 73/311/2 | 28/145/2 | 73/261/1 | 23/16 | 28/16 | 22/145/3 |
| *Enterobacter sakazakii* strain number | 16 | 2 | 2 | 2 | 2 | 0 | 0 | 2 | 2 | 2 | 0 | 0 |
| | 33 | 2 | 2 | 2 | 2 | 2 | 2 | 0 | 0 | 0 | 0 | 0 |
| | 261 | 2 | 2 | 2 | 2 | 2 | 2 | 0 | 2 | 0 | 0 | 0 |
| | 265 | 2 | 2 | 0 | 0 | 2 | 0 | 2 | 2 | 2 | 0 | 0 |
| | 266 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 0 | 2 | 0 | 2 |
| | 269 | 2 | 2 | 2 | 2 | 0 | 2 | 0 | 0 | 0 | 0 | 0 |
| | 270 | 2 | 0 | 0 | 2 | 0 | 2 | 0 | 2 | 0 | 0 | 0 |
| | 271 | 2 | 2 | 2 | 0 | 2 | 0 | 2 | 0 | 0 | 0 | 0 |
| | 272 | 2 | 2 | 2 | 2 | 2 | 2 | 0 | 0 | 0 | 0 | 0 |
| | 273 | 2 | 2 | 2 | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 274 | 2 | 2 | 2 | 0 | 2 | 2 | ? | 0 | 0 | 2 | 2 |
| | 280 | 2 | 0 | 2 | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 281 | 2 | 2 | 2 | 2 | 2 | 0 | 2 | 0 | 0 | 0 | 0 |
| | 284 | 2 | 2 | 2 | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 286 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 0 | 2 | 0 | 0 |
| | 288 | 2 | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 290 | 2 | 2 | 2 | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 292 | 2 | 2 | 2 | 2 | 2 | 2 | 0 | 0 | 0 | 0 | 0 |
| | 297 | 2 | ? | 2 | 2 | ? | 2 | 0 | 0 | 0 | 0 | 0 |
| | 298 | 0 | 2 | 2 | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 300 | 0 | 2 | 2 | 2 | 2 | 2 | 0 | 2 | 0 | 0 | 0 |
| | 303 | 2 | 2 | 0 | 0 | 2 | 0 | 2 | 2 | 2 | 0 | 0 |
| | 305 | 2 | 2 | 2 | 2 | 0 | 2 | 0 | 0 | 0 | 0 | 0 |
| | 308 | 2 | 2 | ? | ? | 0 | 2 | 0 | 0 | 0 | 0 | 0 |
| | 309 | 2 | 2 | ? | ? | 0 | 2 | 0 | 0 | 0 | 0 | 0 |
| | 311 | 2 | 2 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 2 | 0 |
| | 313 | 2 | 2 | 2 | 2 | 2 | 2 | 0 | 0 | 0 | 0 | 0 |
| | 314 | 0 | ? | 2 | 2 | ? | 0 | 2 | 0 | 0 | 0 | 0 |
| | 316 | 0 | 2 | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 318 | 2 | 2 | 2 | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 322 | 2 | 2 | 2 | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 323 | 2 | 2 | 2 | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 1360 | 0 | 0 | 2 | 2 | 2 | 0 | 0 | 2 | 0 | 0 | 0 |
| | 1387/2NL | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 0 | 2 | 2 | 0 |
| | MC7 | 2 | 0 | 0 | 0 | 0 | 2 | 0 | 2 | 0 | 0 | 0 |
| | MC8 | 2 | 2 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | MC9 | 2 | 2 | 0 | 0 | 0 | 2 | 2 | 0 | 0 | 0 | 0 |
| | MM10 | 2 | 0 | 0 | 0 | 0 | 2 | 0 | 2 | 0 | 0 | 0 |
| | MM11 | 2 | 2 | 2 | 2 | 2 | 2 | 0 | 0 | 0 | 2 | 0 |

**TABLE 4: Selection of the phages according to the results obtained in liquid medium. legend: 0 non active; 2 active.**

As may be derived from Table 4, some phage isolate recognize more than 80 % of the 39 *E. sakazakii* strains. Other isolates, however, recognize not even 10 % of the strains tested. The phages isolates that are further active in liquid medium against at least 9 out of the 39 *E. sakazakii* strains selected from the group : FSM-16; FSM-33; FSM-261; FSM-265; FSM-266, FSM-269; FSM-270; FSM-271; FSM-272; FSM-273; FSM-274; FSM-280; FSM-281; FSM-284; FSM-286; FSM-288; FSM-290; FSM-292; FSM-297; FSM-298; FSM-300; FSM-303; FSM-305; FSM-308; FSM-309; FSM-311; FSM-313; FSM-314; FSM-316; FSM-318; FSM-322; FSM-323; FSM-1360; FSM-1387-2NL; FSM-MC7; FSM-MC8; FSM-MC9; FSM-MM10 and FSM-MM11, are selected phage isolates that can be used in the present invention. Preferably, the following 8 phage isolates FSM-Phage 67/33/1 (CNCM I-3130), FSM-Phage F/316 (CNCM I-3131), FSM-Phage 9/261 (CNCM I-3132), and FSM-Phage 73/311/2 (CNCM I-3133), FSM-Phage 73/261(CNCM I-3128), FMS 33/33/1, (CNCM I-3129) FSM-Phage 28/145/2, FSM-Phage 10/261/1 (CNCM I-3127).

It will be appreciated that a skilled person may well examine and select other phage isolates according to the present invention, by subjecting them to the screening tests on solid and liquid medium as detailed above.

**Example 2: Evaluation of bacteriophage cocktail efficacy against a mix of various strains of *Enterobacter sakazakii* in BHI and Reconstituted Infant Formula (RIF)**

In order to evaluate the efficacy of the phage cocktail against *E. sakazakii* strain mixture, the following experiment was carried out.

A mixture of the *E. sakazakii* strains FSM-145, 286, 290, 305 and 1387/2NL as described in example 1 and in P. Breeuwer et al., 2003, *Journal of Applied Microbiology* **95**: 967-973, is used. Individual overnight culture (18h at 30°C) of these strains is diluted 1:1 in fresh BHI and incubated 2h at 30°C. A mix of these strains is carried-out by mixing equal quantities of each stock. Then, the BHI broth or RIF are inoculated with 100 µl of the *E. sakazakii* mix (prepared as described above) so that the final concentration is of about 10 cfu/ml.

The phage isolate cocktail containing FSM_phage 67/33/1 (CNCM I-3130), FSM_phage F/316 (CNCM I-3131), FSM_phage 9/261 (CNCM I-3132) and FSM_phage 73/311/2 (CNCM I-3133) has been tested. For this, a variable amount of each phage is added to the tubes containing *E. sakazakii* mix to reach a final concentration of about 8 log pfu/ml. Negative control without addition of phage (BHI or RIF 9.9ml + *E. sakazakii* mix 100µl) is also included.

All inoculates are incubated at 30°C and samples are taken at various time intervals: 0 - 2 - 4 – 6h. For each sample, the number of bacteria present in the medium was determined by standard plate counting method.

**Results:**

The results are presented in figures 1 and 2. It shows that a bacterial mixture will grow out to high number in the absence of phage cocktail whereas no growth is observed in the presence of the phage cocktail for a period of minimum 6 hours and up to 24 hours.

In conclusion, these experiments demonstrate that adding bacteriophage preparation according to the present invention against *E. sakazakii* to reconstituted infant formulas (RIF) should prevent outgrowth of *E. sakazakii* for at least 6 hours.

**Example 3: Effect on bacteriophage preparation for environment sanitation.**

In order to evaluate the efficacy of the phage cocktail on artificially contaminated surfaces by a *E. sakazakii* strain mixture, the following experiment was carried out.

*Step 1: Preparation of bacteria cells*

A mixture of the *E. sakazakii* strains (FSM-145, 286, 290, 305 and 1387/2NL (see examples 1 and 2)) is used. Individual overnight culture (18h at 30°C) of these strains is centrifuged at 3000rpm for 10min at 4°C. Cell pellet is harvested and diluted in BHI to reach a cell concentration of about 8 log cfu/ml. A mix of these strains is carried-out by mixing equal quantities of each stock.

*Step 2: Adhesion*

Stainless steel disc of $1cm^2$ are plunged in the bacterial solution prepared as described in step 1. Leave at room temperature under gentle shaking for 1h.

*Step 3: Washing and Drying*

To remove unattached cells, stainless steel discs are withdrawn and washed 3 times in Phosphate Buffer solution (PBS: pH 7.0, Merck). The excess of liquid is absorbed with Watman paper, the discs are then dried during 30min under laminar flow. To determine the level of attached cells, step 6 is applied on some discs.

*Step 4: Addition of phage cocktail*

Half of stainless steel discs are plunged in a 8 log pfu/ml phage cocktail solution containing the following phages: FSM_phage 67/33/1 (CNCM I-3130), FSM_phage F/316 (CNCM I-3131), FSM_phage 9/261 (CNCM I-3132) and FSM_phage 73/311/2 (CNCM I-3133). Leave the container at room temperature under gentle shaking for 6 hours. Withdraw some discs at various intervals for bacteria enumeration. In parallel, controls without phage added are carried-out.

*Step 5: Washing*

At defined intervals some discs are withdrawn, washed 3 times in sterile PBS and dry quickly on sterile absorbent paper.

*Step 6: Cell detachment and enumeration*

After step 5, put each disc into tubes containing 9ml TS. Tubes are placed into sonication bath (filled with water and ice mix) for 1min at 50KHz afterwards; tubes are agitated for 10 seconds by using a Vortex. Cell enumeration is performed using a standard plating method.

**Results:**

The results are presented in Figure 3 which demonstrates that the *E. sakazakii* bacteria in the strain mixture (FSMCC # 145, 286, 290, 305, 1987/2NL) can not be recovered anymore from the surface of stainless steel discs treated with $10^8$ pfu/ml phage in the phage cocktail (FSMCC-phage # 67/33/1, F/316, 9/261, 73/311/2), whereas without phage cocktail the bacteria remain viable and can even grow slightly.

In conclusion, the treatment of stainless steel surfaces with at least $10^8$ phages per ml result in killing of the *E. sakazakii* bacteria.

**Claims**

1. A phage isolate with substantial lytic potential towards *E. sakazakii*, having the ability to infect:

i) at least 9 bacterial strains of E. sakazakii from the group consisting of FSM-16 ; FSM-33; FSM-261; FSM-265; FSM-266, FSM-269; FSM-270; FSM-271; FSM-272; FSM-273; FSM-274; FSM-280; FSM-281; FSM-284; FSM-286; FSM-288; FSM-290; FSM-292; FSM-297; FSM-298; FSM-300; FSM-303; FSM-305; FSM-308; FSM-309; FSM-311; FSM-313; FSM-314; FSM-316; FSM-318; FSM-322; FSM-323; FSM-1360; FSM-1387-2NL; FSM-MC7; FSM-MC8; FSM- MC9; FSM-MM10 and FSM-MM11, in a test on solid medium using the spot assay test and,

ii) at least 9 bacterial strains of Esakazakii from the group consisting of FSM-16; FSM-33; FSM-261; FSM-265; FSM-266, FSM-269; FSM-270; FSM-271; FSM- 272; FSM-273; FSM-274; FSM-280; FSM-281; FSM-284; FSM-286; FSM-288; FSM-290; FSM-292; FSM-297; FSM-298; FSM-300; FSM-303; FSM-305 ; FSM-308; FSM-309; FSM-311; FSM-313; FSM-314; FSM-316; FSM-318; FSM-322; FSM-323; FSM-1360; FSM-1387-2NL; FSM-MC7; FSM-MC8; FSM- MC9; FSM-MM10 and FSM-MM11, in liquid medium.

2. A phage isolate according to claim 1, deposited under the reference CNCM I-3127.

3. A phage isolate according to claim 1, deposited under the reference CNCM I-3128.

4. A phage isolate according to claim 1, deposited under the reference CNCM I-3129.

5. A phage isolate according to claim 1, deposited under the reference CNCM I-3130.

6. A phage isolate according to claim 1, deposited under the reference CNCM I-3131.

7. A phage isolate according to claim 1, deposited under the reference CNCM I-3132.

8. A phage isolate according to claim 1, deposited under the reference CNCM I-3133.

9. A phage cocktail, containing at least one phage isolate according to one of claims 1 to 8, which infects at least 80% of *E. sakazakii* strains from the group consisting of FSM-16; FSM-33; FSM-261; FSM-265; FSM-266, FSM-269; FSM-270; FSM-271; FSM-272; FSM-273; FSM-274; FSM-280; FSM-281; FSM-284; FSM-286; FSM-288; FSM-290; FSM-292; FSM-297; FSM-298; FSM-300; FSM-303; FSM-305; FSM-308; FSM-309; FSM-311; FSM-313; FSM-314; FSM-316; FSM-318; FSM-322; FSM-323; FSM-1360; FSM-1387-2NL; FSM-MC7; FSM-MC8; FSM-MC9; FSM-MM10 and FSM-MM11.

10. A food product containing at least one phage isolate or cocktail with substantial lytic potential towards *E. sakazakii*, wherein the phage isolate or cocktail is according to claims 1 to 9.

11. A food product according to claim 10, in the form of a milk based product, a nutritional composition, a pet food, a dietary supplement or any food at risk of contamination by *E. sakazakii*.

12. A food product according to claim 13, wherein themilk based product is a fermented milk, a yoghurt, a curd, a cheese, a fresh cheese, a fermented product, a renneted milk, a drink, a milk based powder, an infant formula or a dried powdered infant formula.

13. A food product according to one of claims 11 to 14, in which the phage isolate or cocktail is in an amount effective to prevent contamination and outgrowth of *E. sakazakii* in said product.

14. A food product according to claim 15, wherein the phage isolate is present in an amount of at least $1.10^4$ pfu per ml, or gram if solid, of the food product.

15. An agent for decontamination of food product by prevention of contamination and outgrowth of *E. sakazakii* and sanitation of factory environment, which comprises an effective amount of at least one phage isolate or cocktail according to one of claims 1 to 9.

16. An agent according to claim 15, wherein the phage isolate is present in an amount of at least $1.10^4$ pfu per ml, or gram if solid.

17. An agent according to claim 15 or 16, wherein the food product is a milk based product, such as a fermented milk, a yoghurt, curd, cheese, fresh cheese, milk based fermented products, renneted milk, drinks, milk based powders, an infant formula, a dried powdered infant formula; a non-milk fermented product; a soy-based product, a nutritional formula, a dairy product, a chilled or shelf stable beverage, water, soup, a dietary supplement, a meal replacement, a nutritional bar or a confectionery, a pet food product, a sweet or sweetened beverage, an ice-cream, a confectionery bar, breakfast cereal flakes or bars or nutritional supplement for clinical nutrition or any food at risk of contamination by *E. sakazakii.*

18. Use of at least one phage isolate or cocktail according to one of claims 1 to 9, for decontamination of food products and sanitation of factory environment.

19. The use according to claim 18, wherein the food product is a milk based product, such as a fermented milk, a yoghurt, a curd, a cheese, a fresh cheese, milk based fermented products, renneted milk, drinks, milk based powders,

an infant formula, a dried powdered infant formula; a non-milk fermented product; a soy-based product, a nutritional formula, a dairy product, a chilled or shelf stable beverage, water, soup, a dietary supplement, a meal replacement, a nutritional bar or a confectionery, a pet food product, a sweet or sweetened beverage, an ice-cream, a confectionery bar, breakfast cereal flakes or bars or nutritional supplement for clinical nutrition or any food at risk of contamination by *E. sakazakii*.

20. Use of at least one phage isolate according to one of claims 1 to 9, in the preparation of a composition intended to prevent or treat infections caused by *E. sakazakii* in humans or animals.

21. The use according to one of claims 18 to 20, in which the phage isolate is present in an amount efficient to prevent contamination and outgrowth of *Enterobacter sakazakii*.

22. The use according to one of claims 18 to 21, wherein the phage isolate or cocktail is used in an amount of at least $1.10^4$ pfu per ml.

23. A method for decontamination of food products or sanitation of factory environment, which comprises using at least one phage isolate or cocktail with substantial lytic potential towards *E. sakazakii*, wherein the phage isolate is according to one of claims 1 to 9.

24. A method for selection of phage isolate with substantial lytic potential towards *E. sakazakii* consisting of screening phage isolate having the ability to infect:

i) at least 9 bacterial strains of *E. sakazakii* from the group consisting of FSM-16; FSM-33; FSM-261; FSM-265; FSM-266, FSM-269; FSM-270; FSM-271; FSM-272; FSM-273; FSM-274; FSM-280; FSM-281; FSM-284; FSM-286; FSM-288; FSM-290; FSM-292; FSM-297; FSM-298; FSM-300; FSM-303; FSM-305; FSM-308; FSM-309; FSM-311; FSM-313; FSM-314; FSM-316; FSM-318; FSM-322; FSM-323; FSM-1360;FSM-1387-2NL ;

FSM-MC7; FSM-MC8 ; FSM- MC9; FSM-MM10 and FSM-MM11, in a test on solid medium using the spot assay test and,

ii) at least 9 bacterial strains of *E. sakazakii* from the group consisting of FSM-16; FSM-33; FSM-261; FSM-265; FSM-266, FSM-269; FSM-270; FSM-271; FSM- 272; FSM-273 ; FSM-274; FSM-280; FSM-281; FSM-284; FSM-286; FSM-288; FSM-290; FSM-292; FSM-297; FSM-298; FSM-300; FSM-303; FSM-305; FSM-308 ; FSM-309; FSM-311; FSM-313; FSM-314; FSM-316; FSM-318 ; FSM-322; FSM-323; FSM-1360; FSM-1387-2NL; FSM-MC7; FSM-MC8; FSM- MC9; FSM-MM10 and FSM-MM11, in liquid medium.