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SENSITIVITY OF SPOILING AND PATHOGEN FOOD-RELATED BACTERIA TO *ORIGANUM VULGARE* L. (LAMIACEAE) ESSENTIAL OIL

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ABSTRACT

Origanum vulgare L. (oregano), Lamiaceae, has been known as plant specie with prominent biological properties for a long time. This study aimed to evaluate the antibacterial activity of *Origanum vulgare* essential oil on various Gram-positive and Gram-negative spoiling and/or pathogen food-related bacteria, as well as to observe its antimicrobial effectiveness in a food conservation micromodel. The results showed a strong antibacterial activity of the assayed essential oil noted by large growth inhibition zones (30-37 mm). MIC values were between 20-40 µL/mL for the most bacteria strains. Essential oil was able to cause significant (P<0.05) inhibitory effect on the bacteria viability providing a bacteriostatic effect after 24hours of exposure. In addition, the MIC provided a significant (P<0.05) decrease of the autochthonous bacterial flora in ground meat samples stored under refrigeration. These results support the possibility of using *Origanum vulgare* essential oil as alternative antimicrobial compound in food conservation systems.

Key words: *Origanum vulgare* L., essential oil, antimicrobial activity, bacteria

INTRODUCTION

Food conservation is based on an intermittent search for foods with a high nutritional quality and microbial stability and it has been reached by the control of the growth/survival of spoiling and pathogen foodborne microorganisms (1,2). For a long time, the microbial safety in foods has been gotten by the use of various physical and/or chemical procedures (6,15). Nowadays, there has been a focus on a decreasing use of chemical preservatives in food conservation because consumers have demanded more natural foods with low impact on the environment characterizing the "green consumerism" (17,19).

This panorama has impelled the research regarding the possible discovery of plant products with antimicrobial properties (30,35). Over 1340 plants are known as potential sources of antimicrobial compounds, however few of them have been scientifically studied (25). Being plant natural foodstuffs, spices appeal to consumers who tend to question the safety of synthetic additives (22). Advantages of spices as antimicrobials in foods are well described and some of their active compounds have been allowed to use in foods (3). Although spices are known for their medicinal, preservative and antioxidant properties, they have been used with primary purpose of enhancing the flavor of foods rather than extending their shelf-life (20,31).

Among many spices that have been scientifically studied regarding their antimicrobial properties, *Origanum vulgare* L. (oregano), Lamiaceae, has showed prominent results (1,14,26). *O. vulgare* is a very versatile plant with many therapeutic properties (e.g. diaphoretic, anti-inflammatory, antiseptic, carminative, antispasmodic and tonic) being used in medicine native systems for a long time (5,24). *Origanum vulgare* essential oil has showed a high content of phenolic compounds which are believed to be responsible for its antimicrobial effectiveness (1,5).

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The present study aimed to evaluate the effectiveness of *O. vulgare* essential oil to inhibit the growth of various Gram positive and Gram negative spoiling and pathogen food-related bacteria. Still, it was assayed the antibacterial effectiveness of *O. vulgare* essential oil in a food micromodel.

MATERIALS AND METHODS

Essential oil

Origanum vulgare essential oil was obtained from Ferquima Ind. e Com. Ltda. (Vargem Grande Paulista, São Paulo, Brazil) and its quality parameters (appearance, color, purity, odor, density - 20ºC, refraction index - 20ºC) were described in an accompanying technical report. This provider produces and commercializes essential oils on industrial scale. The essential oil was assayed at absolute concentration and at concentrations of 160, 80, 40, 20, 10, 5, 2.5, 1.25 and 0.62 µL/mL and the solutions were prepared according to Souza *et al.* (30).

Bacteria strains

Aeromonas hydrophilla INCQS 00318, *Bacillus cereus* ATCC 11778, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 8739, *Enterobacter aerogenes* ATCC 7664, *Klebsiella pneumoniae* ATCC 4362, *Listeria monocytogenes* ATCC 7664, *Pseudomonas aeruginosa* ATCC 9027, *S. aureus* ATCC 6538, *Salmonella choleraesius* ATCC 14028, *S. enterica* ATCC 6017, *Serratia marcencens* ATCC 13880, *Shigella flexneri* MM 412, *S. sonnei* ATCC 11060 and *Yersinia enterocolitica* ATCC 9610 strains were used as test microorganisms. These strains were supplied by National Institute of Quality in Health, FIOCRUZ, Rio de Janeiro, Brazil and Institute of Antibiotics, Federal University of Pernambuco, Recife, Brazil. Stock cultures were maintained on nutrient agar slants at 4ºC. Inocula used in the antimicrobial assays were obtained from overnight cultures on nutrient agar slants at 37ºC and diluted in sterile saline solution (0.85% w/v) to have a final concentration of 106 colony forming unity (cfu)/mL (adjusted according to the turbidity of 0.5 McFarland scale tube).

Antimicrobial assays

Screening: Solid medium diffusion technique using filter paper discs was used for screening the antibacterial activity of *Origanum vulgare* essential oil. For this, 1 mL of the bacterium suspension (approximately 106 cfu/mL) was uniformly spread on sterile agar nutrient Petri dishes. After inoculum absorption by nutrient agar, filter paper discs (Whartman n. 1, diameter 6 mm) were soaked with 20 µL of the essential oil and placed on the inoculated agar (17,24). The system was incubated at 37ºC for 24 hours. At the end of the incubation period, the bacterial growth inhibition zones diameters were measured using calipers and expressed in millimeters. It was considered as positive antibacterial activity when observed growth inhibition zones with diameter equal to or greater than 10 mm diameter. Controls included in this assay were essential oil replaced by sterile water.

Minimum Inhibitory Concentration

MIC determination: Solid medium diffusion procedure using wells in dishes was used to determine the *Origanum vulgare* essential oil MIC. For this, 1mL of the inoculum (approximately 106 cfu/mL) was uniformly spread on sterile nutrient agar Petri dishes. After inoculum absorption by nutrient agar, wells were made using sterile glass tubes (diameter 6 mm) which were filled with 50 µL of the essential oil solutions (160 to 0.62 µL/mL) (22). The system was incubated at 37ºC/24hs. At the end of the incubation period, the MIC was the lowest essential oil concentration showing growth inhibition zones with diameters equal to or greater than 10 mm. Controls included in this assay were essential oil replaced by sterile water.

Kill time study

Kill time study was carried out with the MIC values found previously. For this was used the viable cells count method. 5 mL of double strength nutrient broth was inoculated with 1 mL of the bacterium suspension (approximately 106 cfu/mL). After that, 4 mL of *O. vulgare* essential oil solution, with concentration adjusted to provide an essential oil final concentration similar to the MIC previously determined, was added to the system and followed by shaking for 30s using Vortex. The system was incubated at 37ºC. At different time intervals (1, 2, 4, 8, 12 and 24hs) of exposure, 1mL of the suspension was serially diluted (10-1 - 10-5) in sterile peptone water (0.1% w/v) and inoculated on nutrient agar Petri dishes for 24hs at 37ºC (37). The mean number of colonies (cfu/mL) was counted and compared with that found in the control assay in which the essential oil solution was replaced by sterile distilled water. The results were expressed in log of cfu/mL.

Antimicrobial activity in food micromodel

This experiment was performed in commercially sterile cups of polystyrene (50 cm^3). Initially, 40g of fresh ground meat commercially available were uniformly spread onto the bottom of the cup. After that, 10 mL of the O. vulgare solutions at different concentrations (80, 40 and 20 µL/mL) were uniformly poured on the ground meat. The cup was aseptically sealed and stored under refrigeration (app. 8ºC). 40g of ground meat without essential oil solution addition were used as control assay (11,16). After 24, 48 and 72hs of storage the meat samples were submitted to total bacterial count according to procedure described by Vanderzant and Spplittstoesser (36). Meat samples were weighted and serially diluted (10-1 - 10-6) in sterile peptone water (0.1% w/v). Aliquots of 100 µL were plated on plate count agar for total bacteria count. The system was incubated at 37ºC/24hs for total bacteria count. The counts were given in log of cfu per gram of meat (log cfu/g meat).

All antimicrobial assays were performed twice and the results were expressed as the average of the two repetitions.

Statistical analysis

Statistical analysis was performed to determine significant differences ($P<0.05$) by Tukey test in the bacteria kill time assays and by Student t test in the food micromodel assay. For this was used Sigma stat 2.03 computer program

RESULTS AND DISCUSSION

Table 1 shows the antibacterial activity of *O. vulgare* essential oil on spoiling and pathogen food-related bacteria. The antimicrobial activity is presented by a qualitative and quantitative evaluation expressed in growth inhibition zones diameters and MIC values. All assayed bacteria were sensitive to *O. vulgare* essential oil presenting large growth inhibition zones with diameter between 30-36 mm. Smallest inhibition zones (30 mm) were against *A. hidrophylla*, *B. cereus*, *S. aureus*, *S. enterica*, *S. mercencens*, *S. flexneri* and *Y. enterocolitica* while the largest inhibition zone was against *E. aerogenes* (36 mm). MIC values were between 20-40 µL/mL for the most bacteria strains. Highest MIC value (80 µL/mL) was found against *L. monocytogenes* and *S. choleraesius*. MIC determination procedure showed prominent antibacterial activity of *O. vulgare* essential oil supporting the findings of other authors (9,13,33).

Table 1. Antimicrobial activity of *O. vulgare* L. essential oil against food-related bacteria determined by solid medium diffusion procedure.

Bacteria Strains	Essential oil[a,b] (20µL/disc)	MIC (µL/mL)
A. hidrophylla	30	40
B. cereus	30	20
B. subtilis	31	20
E. aerogenes	36	40
E. coli	32	40
K. pneumoniae	34	40
L. monocytogenes	35	80
P. aeruginosa	33	40
S. aureus	30	20
S. choleraesius	34	80
S. enterica	30	40
S. mercencens	30	20
S. flexneri	30	40
S. sonei	31	20
Y. enterocolitica	30	20

[a] essential oil at absolute concentration; [b] screening results expressed in growth inhibition zones diameters (mm).

Three out from the four Gram positive assayed bacteria showed an MIC value of 20 µL/mL (smallest MIC). Some authors have reported that Gram positive microorganisms are slightly more sensitive to essential oils when compared to Gram negative (4,8). This lower sensitivity of Gram negative organisms has been related to the presence of an outer membrane surrounding their cell wall, which restricts the diffusion of hydrophobic compounds through its lipopolyssacharide covering (34). It has been reported that among Gram negative bacteria, *P. aeruginosa* appears to be least sensitive to essential oils (18,21,38). Therefore, this behavior was not observed in our study, because *P. aeruginosa* showed an MIC value similar to other Gram negative assayed bacteria, as well as did not develop smallest inhibition zones.

Some authors have reported that the simple relation cell structure and microbial sensitivity to essential oils is not yet well established and it could not be taken like a general thought in these interactions. Still, it is proposed that a smaller or higher inhibitory activity of essential oils on Gram positive or Gram negative microorganisms could be related to the particular effectiveness of the essential oil components on different microorganisms rather than to the microbial cell structure (7,9).

Figs. 1 to 4 show the effect of *O. vulgare* essential oil MIC on *S. aureus* (classic Gram positive pathogen), *B. cereus* (spore forming pathogen), *S. enterica* (classic Gram negative pathogen), *Y. enterocolitica* (emerging pathogen), *E. coli* (fecal contamination indicator) and *S. marcences* (food spoiling) viable cells count. As can be seen, the MIC showed significant ($P<0.05$) inhibition of the viability of all assayed bacteria strains. Bacteria counts (cfu/mL) were always smaller than the ones found in the

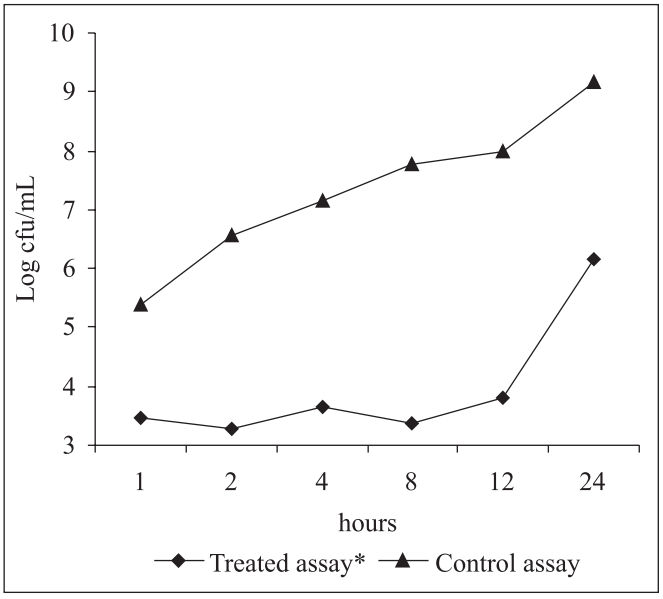

Figure 1. Effect of *O. vulgare* L. essential oil MIC on *S. aureus* viable cells count (*MIC: 20 µL/mL).

control assays. After 24hs of exposure, the treated assays showed counts around 106cfu/mL (next to the initial inoculum) while the control assays showed counts around 109 cfu/mL (100 to 1000 folds higher that the initial inoculum). These findings characterize a bacteriostatic property of *O. vulgare* essential oil on all assayed bacteria and this effect was well marked in the interactions with *E. coli* (Fig. 5) and S. marcencens (Fig. 6) after 12hs of exposure. Bacteriostatic property is characterized when some compound is able to cause the bacteria failure to grow in broth, but are cultured when the broth is plated onto a suitable agar (3,29). Some researches have found strong bactericidal effect (total elimination of microbial initial inoculum) of extracts and *O. vulgare* hydrosols after 72hs of exposure (22,23).

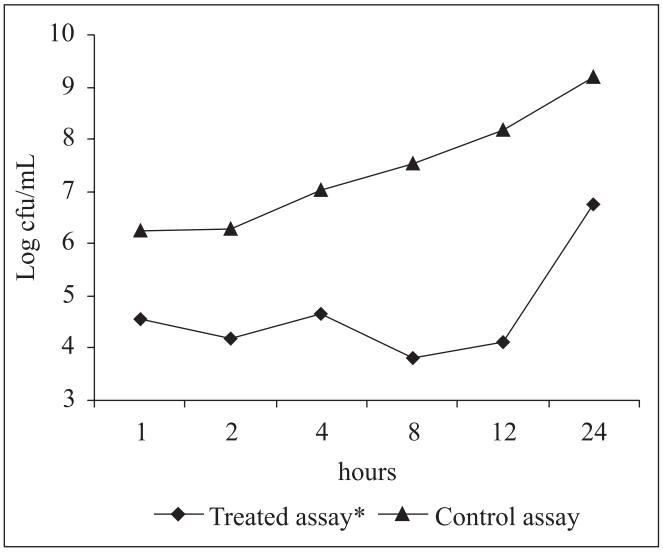

Figure 2. Effect of *O. vulgare* L. essential oil MIC on *B. cereus* viable cells count (*MIC: 20 µL/mL).

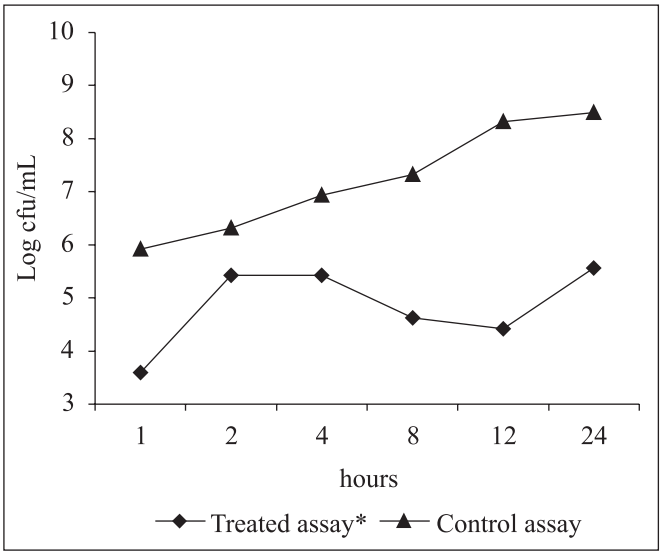

Figure 4. Effect of *O. vulgare* L. essential oil MIC on *Y. enterocolitica* viable cells count (*MIC: 20 µL/mL).

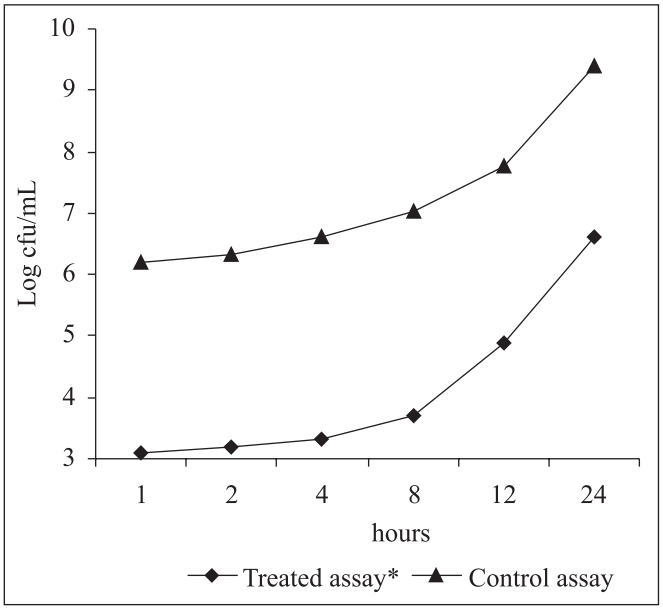

Figure 3. Effect of *O. vulgare* L. essential oil MIC on *S. enterica* viable cells count (*MIC: 40 µL/mL).

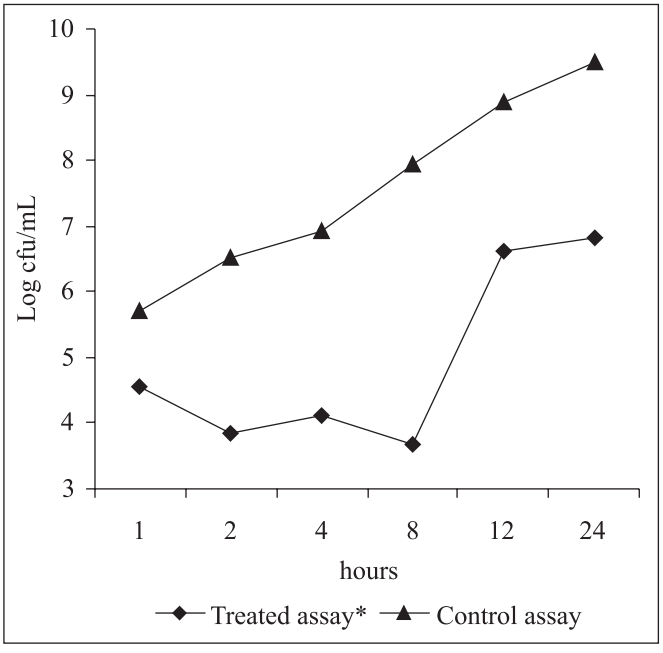

Figure 5. Effect of *O. vulgare* L. essential oil MIC on *E. coli* viable cells count (*MIC: 40 µL/mL).

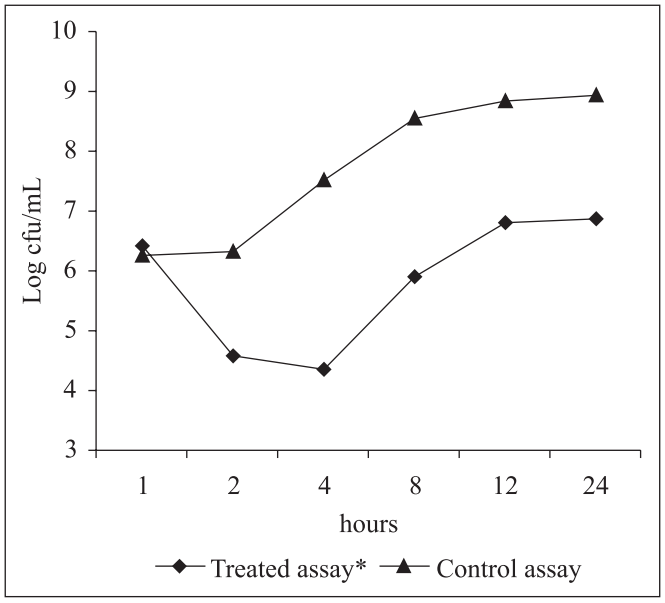

Figure 6. Effect of *O. vulgare* L. essential oil MIC on *S. marcencens* viable cells count (*MIC: 20 µL/mL).

Still, the MIC showed capacity to extend the lag phase of *S. aureus* (Fig. 1) and *B. cereus* (Fig. 2), being noted a trend to establish an exponential growth just after 12 hours of exposure. Valero and Salmerón (35) noted that *O. vulgare* essential oil (25 µg/mL) was effective in extending the lag phase in *Bacillus cereus*.

Table 2 show the inhibitory action of *O. vulgare* essential oil on the total bacteria count in ground meat stored under refrigeration. The different concentrations (80, 40, 20 µL/mL) were chosen based on the MIC values previously found. Antimicrobial performance of *O. vulgare* essential oil in the food micromodel confirmed its antimicrobial potential noted in laboratorial media. Essential oil addition was able to cause statistically significant ($P<0.05$) inhibitory effect on the autochthonous bacterial flora count when compared with the control assay (untreated meat samples). Control assays after 48 hours of storage developed initial decay signs with sensorial changes in color and odor and these changes became more evident after 72 hours (data not showed). On the other hand, treated meat samples did not present decay signs after 72 hours characterizing a stabilizing effect of *O. vulgare essential* oil on the meat sensorial appearance.

Table 2. Effect of *O. vulgare* L. essential oil on total bacteria count (log cfu/g) in ground meat stored under refrigeration.

Storage time(hours)	Essential oil concentration					
	80µL/mL[a]		40 µL/mL[a]		20 µL/mL[a]	
	Treated	Control	Treated	Control	Treated	Control
24	5.32	9.91	6.1	9.0	7.95	8.98
48	7.11	8.65	7.71	8.96	7.84	9.32
72	8.32	9.68	7.86	3.47	8.71	9.86

[a] significant difference ($P<0.05$) between the treated sample and control assay.

The number of researches regarding the antimicrobial activity of spices and derivatives in food matrix still could be considered small when compared to the number of researches using laboratorial media (3,16). Inhibitory effectiveness of *O. vulgare* essential oil in foods has been noted in minced beef (28), beef fillets (32), Asia sea bass (12) and eggplant salad (27).

Our data confirm the antibacterial potential of *O. vulgare* essential oil. In addition, our results support the possibility of its use as alternative antimicrobial to be applied in food bioconservation systems. Therefore, further researches could be carried out to verify its effectiveness in inhibiting the growth/survival of other food related microorganisms, as well evaluating their antimicrobial effectiveness in different foods. Still, it could be interesting a research regarding its toxicological aspects including potential, accumulative and synergistic effects.

RESUMO

Sensibilidade de bactérias deteriorantes e patogênicas de interesse em alimentos ao óleo essencial de *Origanum vulgare* L. (Lamiaceae)

Origanum vulgare L. (orégano), Lamiaceae, tem sido reconhecida como uma espécie vegetal com destacáveis propriedades biológicas por um longo tempo. Este estudo objetivou avaliar a atividade antibacteriana do óleo essencial de *Origanum vulgare* L. sobre várias bactérias Gram positivas e Gram negativas deteriorantes e/ou patogênicas de interesse em alimentos, bem como observar sua efetividade antimicrobiana em um micromodelo de conservação de alimentos. Os resultados mostraram uma considerável atividade antibacteriana do óleo essencial ensaiado notada por grandes zonas de inibição do crescimento bacteriano (30-37 mm). Os valores de CIM encontrados oscilaram entre 20-40 µL/mL para a maioria das bactérias. A CIM do óleo essencial causou um significante ($P<0.05$) efeito inibitório sobre a viabilidade bacteriana, sendo caracterizado uma propriedade bacteriostática após 24 horas de exposição. Ainda, a CIM causou uma diminuição significante ($P<0.05$) da contagem da flora bacteriana autóctone em carne moída armazenada sob refrigeração. Estes dados suportam a possibilidade do uso do óleo essencial de

Origanum vulgare L. como composto antimicrobiano alternativo em sistemas de conservação de alimentos.

Palavras-chave: *Origanum vulgare* L., óleo essencial, atividade antibacteriana, bactérias

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