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ANTAGONISTIC EFFECT OF THE LACTIC BACTERIA ISOLATED FROM THE CAMEL MILK ON STAPHYLOCOCCUS AUREUS IN YOGHOURT MANUFACTURING

This paper deals with the lactic bacteria found in the raw camel milk producing antibacterial substances. Samples of milk were obtained from female camels of herds from nomads living in the south of Algeria. The antibacterial activity of the bacteria was tested on Staphylococcus aureus strains and their role as natural bio-preservatives in the yoghourt was assessed.

Among the seven (07) strains of lactic bacteria which were isolated from camel milk Lb. fermentum (Lc17) presented the highest antagonistic effect on S. aureus. It was verified that there was no interaction between the lactic ferments of yoghourt and Lb. fermentum and no modification of the acidity of the yoghourt. The inhibition of Staphylococcus aureus by yoghurt containing a charge of 5.87 log cfu of Lb. fermentum was compared with that of yoghourt constituted exclusively by lactic ferments, after 4 hours of incubation in a mixed culture.

Key words: antagonism, lactic bacteria, biologic preservatives, camel, *Staphylococcus aureus,* yoghourt.

INTRODUCTION

The lactic acid bacteria enjoy considerable interest because their antagonistic properties make them particularly useful as biological preservatives (Yousef et al., 2003). The microflora of bovine milk has been extensively studied, that of camel milk, instead, has attracted a much restricted attention and just a limited number of studies have been published (Siboukeur 2006).

Many strains of lactic bacteria are currently being studied to evaluate their utility in the prevention or cure of diseases associated with the consumption of yoghourt. Beneficial effects have been detected regarding: digestion of lactose (De Vrese et al., 2001), symptoms of diarrhea (Heyman et al., 2000), inflammatory bowel diseases (Meydani et al., 2000) and, possibly, gastric ulcers (Wang et al., 2004) and the regulation of the blood lipid level (Jacqmain et al., 2003).

OBJECTIVES

The aim of this work was isolating new strains of bacteria from camel milk capable of inhibiting pathogenic bacteria such as *Staphylococcus aureus* and highlighting their antagonistic activities so that they can be applied as biological preservatives in a dairy product: the yoghourt.

MATERIAL AND METHODS

Milk collection

Samples of raw camel milk coming from the Djelfa region (south of Algeria) were collected. Each one was constituted by 150 ml of milk collected directly from the breast of the animal.

Decimal dilutions were prepared using sterile Ringer solution. A volume of 0.1 ml of each dilution was used for double layer surface plating on MRS medium, which was then incubated at 30°C and in the dark for 72h (International Federation of Milk). The reference bacterial strain was *Staphylococcus aureus* ATCC 29213.

Culture media and growth conditions

- MRS agar medium marketed by the Pasteur Institute of Algeria. It was incubated at 30°C during 48 to 72 hours for the growth of lactobacillus.
- Medium Chapman, agar or broth, incubated at 37°C during 24 hours for *Staphylococcus aureus.*
- TSA medium incubated at 37°C during 24 hours.

Purification and identification of lactic acid bacteria

The purification of the isolated bacteria was performed according to the method described by Karam and Karam (1994). Bacteria that showed a Gram positive stain and lacked catalase activity were separated for identification. They were submitted to a series of simple tests for preliminary identification: growth in MRS medium with addition of NaCl, growth at different temperatures and search of the homo- or hetero-fermenting type by the classical method (Petransxiene et al. 1981; Guiraud et al., 1998).

Demonstration of the antagonistic effect of isolates against Staphylococcus aureus

The lactobacillus species were inoculated in tubes containing 9 ml of MRS medium, which were incubated during 18h at 30°C.

Staphylococcus aureus ATCC 29213 was grown in Chapman broth and incubated for 18h at 37°C.

The surface of Petri dishes containing TSA medium was covered with a few milliliters of preliminary culture of *Staphylococcus aureus* strain and allowed to dry for 30 minutes at 37°C.

Discs of blotting paper were soaked with the preliminary culture of lactic acid bacteria. They were left to dry and then placed on TSA media plates which, in turn, were allowed to dry for 30 minutes. Then they were left at 4°C for 4 hours to ensure the diffusion of the substances responsible for the interaction and finally incubated at 37°C for 24 hours.

The inhibition of the indicator strain results in the formation of clear zones around the disks, whose diameter (in mm) is measured from the periphery of the disc (Tadesse et al., 2004).

Effect of the camel milk isolates on the yoghourt ferments

Two flasks with a volume of 110 ml of reconstituted milk, which had been sterilized in the autoclave at 120°C for 20 minutes, were inoculated with the strains of *Lactobacillus delbrueckii* subspecies *bulgaricus* and *Streptococcus thermophilus*, approximately 0.1023 g / l of milk (AFNOR). A volume of 1 ml of camel milk isolates that showed strong inhibitory activity (approximately 5.87 log CFU / ml) against *Staphylococcus aureus* was added to one of the two bottles (bottle 1). Both flasks were incubated at 44°C for 4h.

Growth kinetics

Sampling at increasing time intervals (1h, 2h, 3h, 4h) was followed by the preparation of decimal dilutions in Ringer solution. Then 0.1 ml of the appropriate dilution was plated on MRS medium for enumeration of *Lactobacillus delbrueckii* subspecies *bulgaricus* ferment and M17 for *Streptococcus thermophilus* ferment count.

After studying the characteristics that differentiate the species of lactic acid bacteria (isolates of camel milk and yoghourt ferment *Lactobacillus delbrueckii* subspecies *bulgaricus*) belonging to the same genus *Lactobacillus,* growth in the presence of 6.5% NaCl was shown specific for the isolate but not for the lactic ferment of the yoghourt.

In order to eliminate any confusion, the count of camel milk isolate was performed on MRS medium with addition of 6.5% NaCl. The acidity produced by pure strains was assessed by titration, sampling 10 ml from each bottle (Mom et al., 2010).

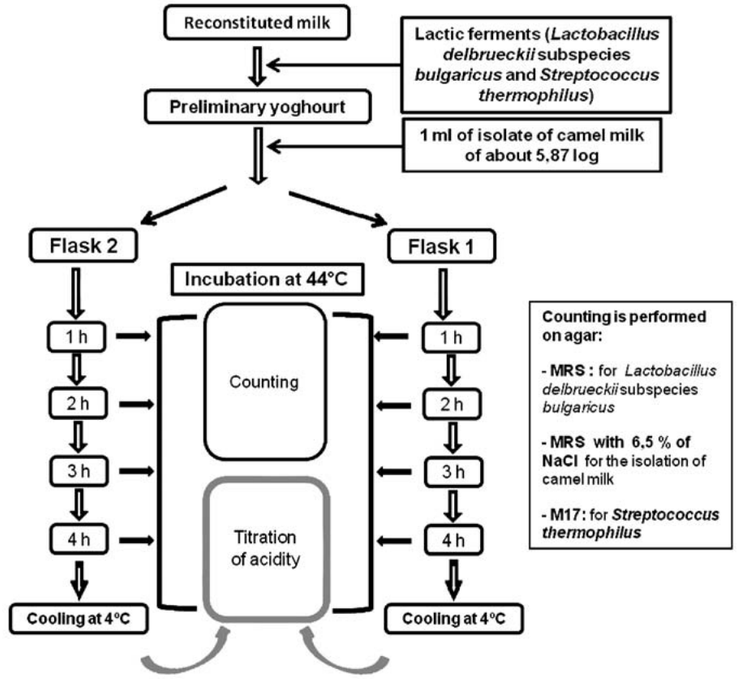


Figure 1. Experimental proceeding for the preparation o a yoghourt to which the isolate of camel milk.

Effect of the camel milk isolates on the behavior of *Staphylococcus aureus* in the yoghourt

Preliminary culture preparation

With each isolate of camel milk and *Staphylococcus aureus* were inoculated 10 ml of sterile skim milk. The preliminary cultures were prepared by incubation at 30°C until coagulation.

Preparation of the preliminary yoghourt

The preliminary yoghourt was prepared from reconstituted milk sterilized by autoclaving at 120°C for 20 minutes and inoculated with 0.01125 g / ml of lyophilized ferments.

The preliminary yoghourt was then dosed in 2 bottles:

Bottle 1 was inoculated with 1 ml of *Staphylococcus aureus* from about 5.05 log CFU / ml.

Bottle 2 was inoculated with 1 ml of *Staphylococcus aureus* from about 5.05 log CFU / ml and 1 ml of isolates of approximately 5.87 log CFU / ml.

Both flasks were incubated at the same time at 37°C for 5 h to achieve maximum growth of *Staphylococcus aureus*.

Samples were taken at regular intervals (1h, 2h, 3h, 4h, 5h) and decimal dilutions (10^{-1} to 10^{-4}) from the bottles were performed in order to make a count of *Staphylococcus aureus* on medium Chapman, which was then incubated at 37°C for 24 h.

Furthermore 10 ml extractions were performed to measure pH.

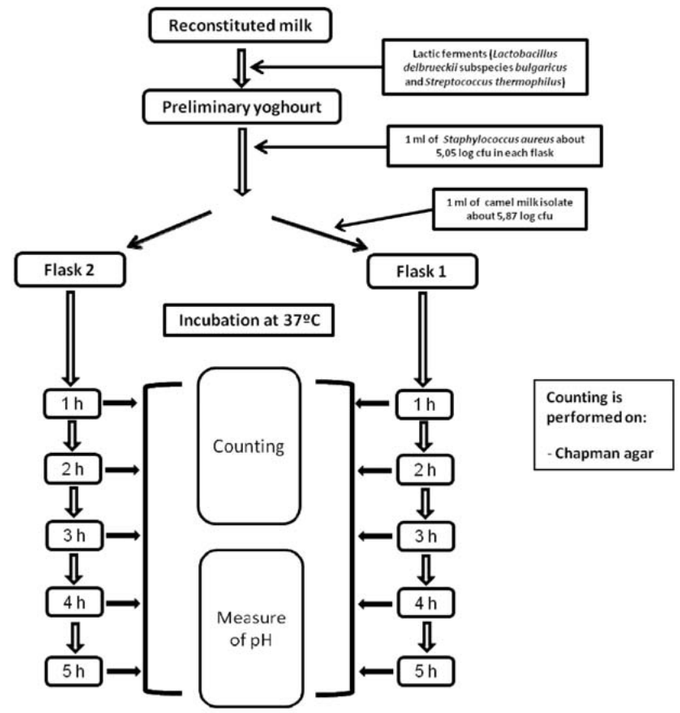


Figure 2. Diagram showing the preparation of a contaminated experimental yoghourt.

RESULTS

Characterization of isolates

The physiological and biochemical characteristics of the isolates are shown in the table below.

Among the 23 strains isolated from camel milk, 7 isolates were taken into account, all being Gram +, motionless, catalase negative and possessing bacillary form.

Strains LC4, LC16, Lc17 and Lc22 of Lactobacillus are classified within the group Betabacterium because they produce CO2 on glycoside medium.

Strain LC4 shows growth at 10°C and is close to the species *Lactobacillus viridiscens.*

Strain LC16 develops at 45°C and ferments arabinose and raffinose, being close to the species *Lactobacillus buchneri*, while Lc17 hydrolyzes arginine and is close to the species *Lactobacillus fermentum.*

УКРАЇНСЬКИЙ БІОФАРМАЦЕВТИЧНИЙ ЖУРНАЛ, № 4(27) 2013

Table I

PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF THE ISOLATES FROM CAMEL MILK.

Characteristic / Lc2		Isolate Lc3	Lc4	Lc7	Lc16	Lc17	Lc22	
Gram		-	-	-	-	-	-	-
Catalase		-	-	-	-	-	-	-
Morphology		bacillus	bacillus	bacillus	bacillus	bacillus	bacillus	bacillus
CO2 on glucose		-	-	+	-	+	+	-
Growth at 45°C		+	+	+	+	+	+	-
Growth at 10°C		-	-	+	-	+	+	+
Growth with NaCl	**2%**	+	+	+	+	+	+	+
	3%	+	+	+	+	+	+	+
	6,5%	+	+	+	+	+	+	+
ADH		-	-	-	+	+	+	+
Esculine hydrolysis		+	-	-	-	-	-	+
Survival at 60°C / 30 minutes		+	+	+	+	-	+	+
Glucose		+	-	-	+	+/-	+	+/-
Raffinose		+	-	-	+	+	+	+
Lactose		+	+	-	+	+	+	+
Cellubiose		+	-	-	+	+	+	+
Mannitol		+	-	-	+	+	+	+
Arabinose		+	-	-	+	+	+	+
Xylose		-	-	-	+	-	+	+

The streptobacterium group, which differs from the preceding one because there is no production of gas, is represented by strain Lc22, which grows at 10°C but not at 45°C.

Strain Lc22 ferments the group of sugars which were tested and is close to the species *Lactobacillus plantarum.*

*S*trains LC2, LC3 and LC7 grow at 45°C but not at 10°C and do not produce CO_2, and thus they are classified into the group Thermobacterium.

Strain Lc2 ferments the raffinose, hydrolyzes esculin and is close to *Lactobacillus acidophilus*, while strain Lc3 ferments lactose and is close to the species *Lactobacillus helveticus*.

Strain Lc77 hydrolyzes arginine and is close to the species *Lactobacillus delbrueckii* subsp *lactis*.

The large number of isolates which were obtained can be explained because the bacteria were incubated for 5, 7, 9 and 16 days.

Antagonistic effect of camel milk isolates on *Staphylococcus aureus*

The diameter registered for the inhibition zones varied from 07 mm (strain Lc2 showed the lowest activity) to 13 mm (the highest activity was recorded for strain Lc17).

Table 2

DIAMETER OF THE INHIBITION ZONES OF THE 7 ISOLATES FROM THE CAMEL MILK IN FRONT OF STAPHYLOCOCCUS AUREUS BY THE METHOD OF DIFFUSION ON TSA MEDIUM.

Strain		TSA
Lc 2	*Lactobacillus acidophilus*	**07 mm**
Lc 3	*Lactobacillus helveticus*	**11 mm**
Lc 4	*Lactobacillus viridiscens*	**00 mm** (no inhibition)
Lc 7	*Lactobacillus delbrueckii* subsp *lactis*	**11 mm**
Lc 16	*Lactobacillus buchneri*	**11 mm**

Continuation of table 2

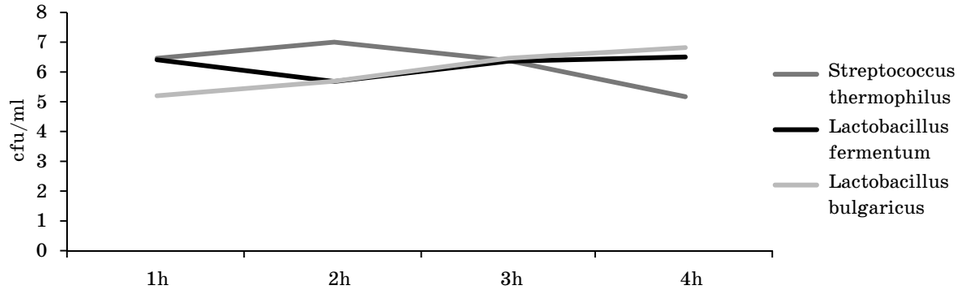
Lc 17	*Lactobacillus fermentum*	**13 mm**
Lc 22	*Lactobacillus plantarum*	**10 mm**

Interactions between *Lactobacillus fermentum* and yoghourt ferments

Count of yoghourt ferment and *Lactobacillus fermentum* after 4 hours in mixed culture

The growth of lactic acid bacteria was stationary after 4 hours of incubation both in camel milk (*Lactobacillus fermentum*) and in the yoghourt (*Lactobacillus fermentum* and *Lactobacillus delbrueckii* subspecies *bulgaricus* and *Streptococcus thermophilus*).

The number of lactic acid bacteria varied between 7.00 and 5.17 log CFU / ml.

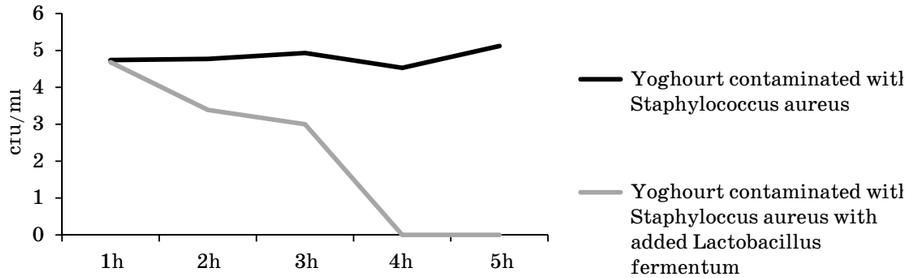


Growth kinetic of *Lactobacillus fermentumet, Lactobacillus delbrueckii* subspecies bulgaricus and *Streptococcus thermophilus* in a mixed culture in the yoghourt

Figure 5. Growth kinetic of *Lactobacillus fermentumet, Lactobacillus delbrueckii* subspecies bulgaricus and *Streptococcus thermophilus* in a mixed culture in the yoghourt.

The addition of *Lactobacillus fermentum* isolated from camel milk has no inhibitory effect against yoghourt ferments (*Lactobacillus delbrueckii* subspecies *bulgaricus* and *Streptococcus thermophilus*).

Biochemical analysis After 4 h of incubation the acidity increased significantly, reaching 58°D in the yoghourt which was inoculated with *Lactobacillus fermentum*. After cooling, as a means for interrupting fermentation, the acidity values of both types complied with AFNOR standards.



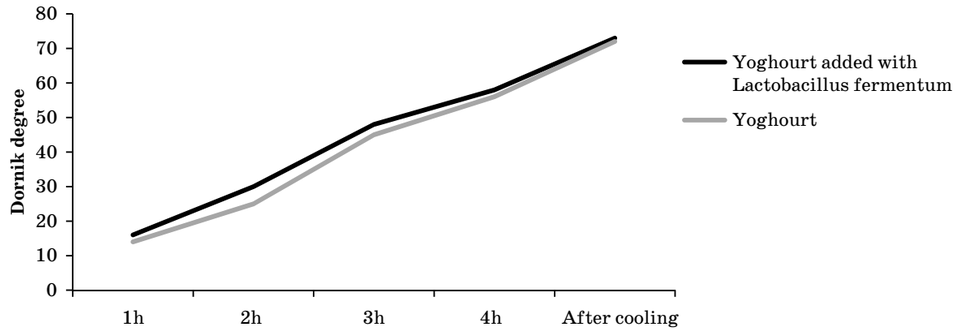
Growth kinetic of *Staphylococcus aureus* in isolated and mixed culture with *Lactobacillus fermentum*

Figure 6. Growth kinetic of *Staphylococcus aureus* in an isolated and mixed culture with L*actobacillus fermentum*

Effect of *Lactobacillus fermentum* on the behavior of *Staphylococcus aureus* in the yoghourt

It was not detected any antagonist effect on *Staphylococcus aureus* which was incorporated at about 5.05 cfu / ml in bottle 1 (yoghourt without *Lactobacillus fermentum*). By contrast, after addition of *Lactobacillus fermentum*, a decrease was detected after only 2 hours of incubation and after 4 hours there is a lack of growth of *Staphylococcus aureus*, which demonstrates the inhibitory effect of *Lactobacillus fermentum*.

If a high burden of *Staphylococcus aureus* (> 5.5 log CFU / ml) is introduced, the effect of *Lactobacillus fermentum* is not noticeable.

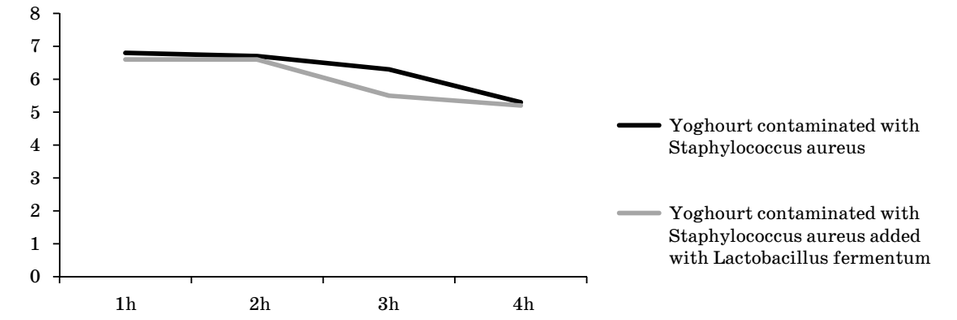


Evolution of the acidity during the maturation of both types of yoghourt expressead in Dornik dregrees

Figure 7. Evolution of the acidity during the maturation of both types of yoghourt expressed in Dornik degrees.

Biochemical analysis

Whereas pH is stable in both types of yoghourt during the 2 hours of incubation, afterwards, a sharper decrease of pH is detected for both types of yoghourt.



Evolution of pH during the maturation of the two types of yoghourt contaminated with Staphylococcus aureus

Figure 8. Evolution of pH during the maturation of the two types of yoghourt contaminated with *Staphylococcus aureus.*

The final pH is a little high, since it has a value of 5.2 in comparison with the pH of common yoghourt which is 4.34. This is a consequence of the incubation temperature (37°C), which is lower than the temperature of maturation of the yoghourt (44°C).

DISCUSSION

The seven isolates were all Gram +, motionless and catalase negative, confirming the data of Gunter et al., 1998.

The fermentation profile of the seven *Lactobacillus* isolates was compared with the reference strains as exposed in Bergey's key (1986). Significant differences were found. For example: *Lactobacillus fermentum* differs from the strain reference because of the fermentation of arabinose and mannitol.

The identified species *Lactobacillus plantarum* is only represented by the strain Lc22 while *Lactobacillus* strains isolated by Karam et al. 1995 belong essentially to the *species plantarum*.

The addition of *Lactobacillus fermentum* isolated from camel milk has no inhibitory effect against yoghourt ferments (*Lactobacillus delbrueckii subspecies bulgaricus* and *Streptococcus thermophilus*).

The acidity of the yoghourt where *Lactobacillus fermentum* was added meets AFNOR standards. The decrease in the number of living cells of *Staphylococcus aureus* from the 3rd hour of incubation in the presence of *Lactobacillus fermentum* shows the inhibitory effect of the latter. This result has been observed in the work of Mami et al., 2010 in some species of *Lactobacillus* on *Staphylococcus*

aureus. The results show a total lack of *Staphylococcus aureus* in the presence of *Lactobacillus fermentum* after only 4 hours of incubation in mixed cultures, whereas this was only observed by Mami et *al.*, 2010 after 72 hours.

The inhibitory effect of the *Lactobacillus fermentum* strain of *Staphylococcus aureus* at a concentration of up to 5.05 log CFU is nil.

In any case, the inhibition of Staphylococcus aureus is not caused by pH, because in the course of maturation the yoghourt contaminated with *Staphylococcus aureus* and without *Lactobacillus fermentum* has 3.90 log CFU of *Staphylococcus aureus* and the pH is 5.3 after 4 hours of incubation. Conversely, in the yoghourt contaminated with *Staphylococcus aureus* and to which *Lactobacillus fermentum* is added there is a total absence of *Staphylococcus aureus* with a similar value of pH, i.e. 5.2.

Inhibition of *Staphylococcus aureus* caused by the production of hydrogen peroxide must be excluded because *Staphylococcus aureus* has a catalase.

Competition for the amino acids in the milk medium cannot explain the inhibition of *Staphylococcus aureus*, because it grows in the milk without using aminoacids (Alomar et al., 2007).

CONCLUSION

Camel milk contains lactic acid bacteria possessing interesting inhibitory potential on pathogens (*Staphylococcus aureus*). In this sense, our study was carried out on a lactic strain represented by *Lactobacillus fermentum* isolated from the camel milk. It has been found that a dose of 5.87 log CFU / ml of *Lactobacillus fermentum* isolated from camel milk achieved an almost total inhibition of *Staphylococcus aureus* after only 5 hours of incubation. It should be pointed out that there was neither change in the physico-chemical parameters which were studied (pH, acidity) in the yoghourt to which *Lactobacillus fermentum* was added, nor any influence on their ferments.

And finally, the antagonistic effect of *Lactobacillus fermentum* against *Staphylococcus aureus* observed in our study shows the possibility of using the former as biological preservative for food.

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OUTSTRANDING PHRASES

The addition of *Lactobacillus fermentum* isolated from camel milk has no inhibitory effect on yoghourt ferments

The decrease in the number of living cells of *Staphylococcus aureus* from the 3rd hour of incubation in the presence of *Lactobacillus fermentum* shows the inhibitory effect of the latter.

Camel milk contains lactic acid bacteria possessing interesting inhibitory potential on pathogens (*Staphylococcus aureus*).

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