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Studies on Biocoating Oral Immunization of *Oreochromis niliticus* Fingerlings with *Aeromonas hydrophila* Bacterin

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ABSTRACT

Oral vaccination is a very easy vaccine administration method with no stress to the immunized fish but it should be in formulation protect antigen containing from inactivation or digestion during passage through foregut and hindgut. In the present study artemia nauplii was used as bio-encapsulation for *Aeromonas hydrophila* bacterin to immunize tilapia fingerling via oral route comparing with immersion and intra-peritoneum routes. The relative percent of survival in tilapia groups vaccinated by oral, immersion and intra-peritoneum routes was 92.68%, 81.4% and 87.5% respectively. The humoral immune response of immunized fish was investigated by micro titre agglutination test showed end point 400, 400 and 800 for oral, immersion and intra-peritoneum routes. And investigated by hemagglutination inhibition test that showed end point 128, 128 and 256 respectively. Also, immune response was measured by ELISA that showed similarly optic density between oral and immersion immunization routes. In conclusion, the current trial for fish vaccination showed that the coated oral vaccine can be effectively used in diseases prevention programs.

INTRODUCTION

Disease is one of the most important limiting factors in aquaculture. Optimal husbandry and general management including biosecurity nutrition, genetics, system management and water quality are critical for maximizing aquatic animal health. However, all facilities are vulnerable to disease outbreaks because many pathogenic organisms are opportunistic and present in the environment, or may be found on some fish that are not showing signs of disease *(1)*.

Prophylactic treatments and good management practices can usually prevent or reduce the susceptibility to diseases. Although, antibiotics can overcome bacterial diseases, consumer health and food safety issues prevent their use in aquaculture. Moreover, established viral diseases cannot be treated. Therefore, vaccination is the best alternative to combat bacterial and viral diseases. It has been a key tool in the success of the livestock industries in the fight against infectious diseases *(2)*.

In salmon farming, the use of vaccines is now used, so that all fish stocked in sea cages are vaccinated. Vaccine has been proven to be cost effective and it is the key reason for the success of that industry. However, in Asia, fish vaccination is still a new concept that is not very well known and understood among farmers and professionals working in the area *(3)*.

The use of vaccines, combined with good health management techniques, may result in substantial disease prevention and so production becomes more predictable. Vaccines are a preventative measure as opposed to antibiotic treatment which is used after a disease outbreak. But vaccines for aquaculture unlike antibiotics, which kill or stop disease causing bacteria, vaccines stimulate the fish's immune system to produce

antibodies that help and protect the fish from diseases *(4)*.

With oral vaccination, the vaccine is either mixed with the feed, coated on top of the feed or bio-encapsulated. When antigens are to be incorporated in feed, the heat sensitivity of the antigen has to be considered and when vaccines are used as top dressing in feed, a coating agent is usually applied, either to prevent leaching of the antigen from the pellets or to prevent breakdown of the antigen in the acidic environment of the fish foregut *(5)*.

Bio-encapsulation is used where fish fry are to be vaccinated and in this case, live feed, as artemia nauplii are incubated in a vaccine suspension after which they are fed to the fry. Since these live organisms are non-selective filter feeders, they will accumulate the antigen in their digestive tract and as such transform themselves into living microcapsules *(6)*.

The aim of the present work was to determine whether of the biocoater oral immunization of *Oreochromis niliticus* with *Aeromonas hydrophila* (*A. hydrophila)* can elicit systemic immune responses and can enhance protection compared to other immunization routes.

MATERIAL AND METHODS

Fish

Fingerlings of tilapia species were obtained from Central Laboratory for Aquaculture Research (CLAR) farm with an average body weight of 10 gm ±2.The fish were kept in 1000 L aquaria supplied with aerated fresh water and fed with commercial pelleted diet twice a day. The water temperature was kept at 25 ± 1 °C. The experimental fish were acclimatized in the experimental aquaria for 2 weeks prior to vaccination.

Glass Aquaria

Glass aquaria of dimensions (80×40×40 cm) were used for the experimental purpose.

Artemia Hatching

Artemia cysts (Bio- Marine Brand) were hatched in transparent cylinder-conical containers. The air was supplied through open aeration line down to the tip of the conical part of the aquarium and the oxygen level was maintained about 4 mg/l. Natural sea water was used as hatching medium. The temperature was adjusted at 28 °C and pH at 8-8.5. Illumination was adjusted at 2000 lux at the water surface. The artemia cysts were incubated at density of 2 gm/l for 24 hr. After hatching, the aeration was stopped and harvested the newly hatched nauplii from the bottom of the container *(7)*.

Bacterial Strain and Growth Conditions

Preparation of *A. hydrophila* kindly obtained from Fish Diseases and Management Department of CLAR, then inoculated into tryptic soy broth (TSB) and incubated for 24h at 25°C.

Virulent Evaluation of (LD_{50}) for *A. hydrophila*

LD_{50} of obtained *A. hydrophila* was tested using 60 Nile tilapia 10 gm ±2 which equally divided into 6 groups. The fishes were kept in 6 glass aquaria (80×40×40 cm of each one) at (25 ± 1) °C. *A. hydrophila* was estimated with Mcfarland barium sulphate standard tube (Difico) at 2× 10^8 cfu/ml and injected 0.2ml intra-peritoneum (I/P), intra-muscular (I/M) (two replicates for each one) and two replicates represented as control. The inoculated fishes were observed for 21 days for recorded the mortality and disease symptoms *(8)*.

Vaccine Preparation

Formalin (40% w/v) was added to the broth culture at a final concentration of (0.5% V/V) and left 48 hrs at room temperature. The inactivated cells were harvested by centrifugation at 4000 xg for 10 min., then

washed twice in 0.3% formalized PBS (phosphate buffer saline) and re-suspended to the density of LD_{50} resulting in 50% mortality *(9)*.

Sterility Test

The prepared vaccine was tested for sterility from bacterial contamination using tryptic soya agar, brain heart infusion agar, blood agar media and Mac Conky agar (Difico). Also, it was tested for mycotic and mycoplasma contamination which was conducted using Sabarout dextrose agar and mycoplasma selective media *(10)*.

Safety Test

Six groups of Nile tilapia fingerling, 30 individuals each, were used in the safety test experiment. Every group was taken 0.2ml bacterin, while control groups were taken saline by different routes *(10)* as showed in Table 1.

Table 1. Safety Test

Group	Fish No.	Used M.O	Average Dose	Inoculated Route
1	30	*A. hydrophila*	0.2ml	Oral
2	30	*A. hydrophila*	0.2ml	Immersion
3	30	*A. hydrophila*	0.2ml	I/P
4	30	Saline	0.2ml	Oral
5	30	Saline	0.2ml	Immersion
6	30	Saline	0.2ml	I/P

Vaccination Procedures

Three groups of 45 fingerlings of tilapia each were used for three routes of vaccination (oral, immersion and intra-peritoneum) in addition to the 3 control groups represented by 15 fish per group as showed in Table 2. The vaccinated and control fish were kept equally in glass aquaria with de-chlorinated tap water and supplied with air pumps.

Table 2. Vaccination Procedures

Group	Fish No.	Used M.O	Inoculated Route
1	45	*A. hydrophila*	Oral
2	45	*A. hydrophila*	Immersion
3	45	*A. hydrophila*	I/P
4	45	Saline	Oral
5	45	Saline	Immersion
6	45	Saline	I/P

Before vaccination, blood was collected and pooled from a random fish samples per group (control and vaccinated groups) by caudal vein puncture to confirm that the fish were free from *A. hydrophila* antibody. Each group was kept isolated in a separate aquarium to prevent cross-infection during the whole trial period. All groups of fish were provided with the same type of balanced feed. Booster dose was applied after 2 weeks from the 1st one with the same techniques.

Oral Vaccination

The harvested newly hatched artemia nauplii are incubated in a suspension of formalized whole culture *A. hydrophila* at 28°C for 24hr in concentration 1ml bacterin with 200 nauplii. The oral vaccine was given in the form of living nauplii fed with bacterin to tilapia finagling in average rate 200 nauplii to 5 fingerling as 0.2 ml bacterin/ fingerling *(6)*.

Immersion Vaccination

Fish were immersed for 30 min in diluted vaccine in a separate vaccine aquaria in form 1 volume of vaccine to 10 volumes of aquarium water with adjust dose to 0.2 ml bacterin / fingerling. The fish were removed carefully from the vaccinating aquarium and then returned to their original after vaccination. The technique was done *(4)*.

Intra-peritoneum Vaccination

Fingerling was inoculated intra-peritoneum by 0.2 ml / fingerling. The process was repeated until the vaccination of all fish group was completed *(11)*.

Challenge Test

Four groups of 50 fingerlings of tilapia fish were used for three routes of vaccination oral, immersion and intra-peritoneum in addition to the control group represented by 50 fish per group. Challenge was applied on vaccinated and control groups after 4 weeks from vaccination day by bath technique *(12)* in which *A. hydrophila* was inoculated in 500 ml of tryptic soy broth for 24 hr at 25°C. The cultures (1 volume) were added to 10 volumes of the aquarium water for each group. The challenge process persisted for one hour. Fish were transferred to their original aquaria and observed for one week post challenge for any clinical abnormalities and mortalities.

The level of protection was calculated as Relative Percent Survival *(11)*.

(RPS) % = 1- (percent of immunized mortality/ percent of control mortality) × 100

Blood Samples

Fish were anaesthetized with MS222 (100 mg/l). Blood samples were collected from the caudal vein from the fish groups before immunization (zero day), after vaccination by 4 weeks and after challenge by 6 weeks from zero day. The collected blood allowed clotting at 4°C for 4 hr. After centrifugation, serum was removed and frozen at -20°C until use.

Preparation of Hyper Immune Serum

Hyper Immune Serum against *A. hydrophila* was prepared in New Zealand rabbits according to *(13)*. Sera were collected and stored at - 20°C until use as positive control in immune response evaluation.

Evaluation of Tilapia Fingerling Immune Response

Micro titre agglutination

Serial dilutions of antibody are prepared in 0.5 ml volumes in test tubes and the same volume of *A. hydrophila* 2×10^8 cells/ml is added to the tubes and mixed. Instead of test tubes microplates having 96 wells are used; 50µl of saline was taken in each well of 96 well micro-titre plates. Serial doubling dilutions of the serum are prepared by adding 50µl serum to first well, mixing and transferring 50 µl to the second well, continuing to the third and so on. A saline control is also set up to 50 µl bacterial suspension is added to each well, mixed and plates are incubated overnight at ambient temperature in moist chamber *(11)*.

Haemagglutination Inhibition Test

Sera were diluted in PBS from 1:2 to 1:512 in U-shaped 96 -well microtitre plate. Non-specific inhibitors were removed by the treatment of sera before testing by heat inactivation. Non-specific agglutinin for erythrocytes was removed by the addition of erythrocytes to the sera prior to testing. This procedure was carried out for each serum before testing. 4HA of antigen is added to each well containing diluted tested sera except for the serum control wells. A back titration of

antigen was incorporated into the test from 4 HA units to 0.25 HA units. The plate is then allowed to stand at room temperature for 60 minutes after which 0.4% chick cells are added to each well and incubated at 4°C for 60 minutes *(14)*.

Enzyme-Linked Immunosorbent Assay (ELISA)

Specific antibodies against *A. hydrophila* for the immunized groups were determined by ELISA ***(15)***. Suspensions of formalin-killed *A. hydrophila* cells (200 µl of 10 µg of cell protein/ml) in phosphate buffer (0.1mol/ l, pH 7.5) were placed in 96-well microtiter plates. The plates were left overnight at 4°C and were washed three times with PBS containing 0.1% Tween-20 (washing solution). 100µl of PBS containing 0.5% bovine serum albumin (BSA) as blocking solution was added to each well. Following incubation for 60 min at 25°C, the wells were washed with the washing solution and 50 µl of serial two-fold dilution of tested serum, and 50 µl of anti-tilapia IgM rabbit IgG (Shanghai Shenggong, China) (1 : 100 dilution in PBS) was added. After incubation for 90 min. at 25°C, the wells were washed with the washing solution, and 50 µl of horseradish peroxidase conjugated anti-rabbit IgG (1: 8000 dilution in PBS) was added.

The plates were then incubated for 90 min. at 25°C and washed. Next, 100 µl of substrate solution (0.4 mg/ml o-phenylenediamine and 0.2 µl ml of 30 % H_2O_2 in citrate buffer) was added and left to react for 10 min at 25 C. The enzyme reaction was stopped by adding 100 µl 2N H_2SO_4 , and the absorbance at 490 nm was measured with a microplate reader (Bio-Tek, USA). Antibody titers were presented as the reciprocal of endpoint dilution exhibiting A_{490} more than three times that of background.

RESULTS

Virulent Evaluation of (LD_{50}) for *A. hydrophila*

The collective mortality was recorded in Table 3.

Table 3. Collective Mortality of Virulent Evaluation of *A. hydrophila*

Group	Fish No.	Inoculated M.O	Inoculated Route	Mortality	Percent
1	10	*A. hydrophila*	I/P	10	100
2	10	*A. hydrophila*	I/P	9	90
3	10	*A. hydrophila*	I/M	9	90
4	10	*A. hydrophila*	I/M	9	90
5	10	Saline	I/P	----	----
6	10	Saline	I/M	----	----

Sterility test

The bacterin was tested for their sterility by cultured on bacterial, fungal and mycoplasma media that was showed no growth on them.

Safety Test

Oral vaccinated fingerling didn't show post vaccinal reaction for 8 weeks. Immersion vaccinated fingerling showed mild hyperemia in gills and some eye reddens. Interperitoneum injected fingerling showed moderate skin lesion at site of inoculation and off food for 2 days after vaccination. Control groups were showed no vaccination reaction.

Survival Rate of Vaccinated Tilapia after Challenge

The level of protection was calculated by Relative Percent Survival as illustrated in Table 4. Re-isolation of the pathogen was from kidney tissues of moribund and fresh dead fish groups that showed the same inoculated bacteria of *A. hydrophila*.

Table 4. Mortality and Survival of Vaccinated Tilapia after Challenge

Vaccination Route	Mortality		Survival		RPS
	Vaccinated	Control	Vaccinated	Control	
Oral	3 (6%)	41 (82%)	47 (94%)	9 (18%)	92.68%
Immersion	8 (16%)	43 (86%)	42 (84%)	7 (14%)	81.4%
Intra-peritoneum	5 (10%)	40 (80%)	45 (90%)	10 (20%)	87.5%

Evaluation of Tilapia Fish Immune Response

Micro titre agglutination

The reciprocal of the antiserum dilution at the end point in micro titre agglutination test for fish groups before immunization, after immunization and after challenge at the previewed times showed in Table 5.

Table 5. Micro titre agglutination

Vaccination Route	Before Immunization	After 4 weeks	After 6 weeks
Oral	-----	400	100
Immersion	-----	400	100
Intra-Peritoneum	-----	800	200
Control	-----	-----	-----

Haemagglutination Inhibition Test

The obtained cut point by hemagglutination inhibition test for fish groups before immunization, after immunization and after challenge showed in Table 6.

Table 6. Haemagglutination Inhibition Test

Vaccination Route	Before Immunization	After 4 weeks	After 6 weeks
Oral	-----	128	32
Immersion	-----	128	32
Intra-Peritoneum	-----	256	64
Control	-----	-----	-----

Enzyme-Linked Immunosorbent Assay

The ELISA representing the antibody titres of fish serum during the vaccination period showed high antibody titre when compared with the control fish serum or zero day fish serum. This antibody level gradually decreased after challenge at 6th weeks postvaccination (Fig.1).

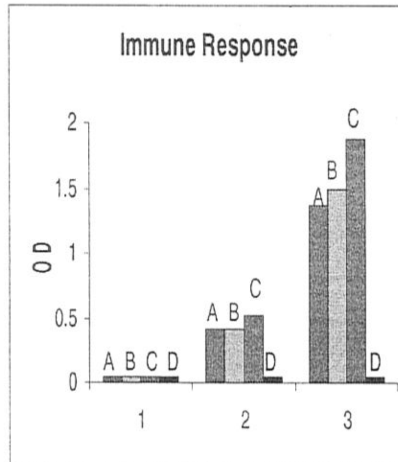

Fig.1: Immune Response of Oral, Immersion and Intra-peritoneum Immunized Fish Groups.
(A) Oral (B) Immersion (C) Intra-peritoneum (D) Control
1. Before Vaccination 2. after vaccination by 4 weeks 3. after vaccination by 6 weeks

DISCUSSION

Orally delivered pharmaceutically active agents present a significant problem in transiting fish's foregut; an organ whose contents represent a harsh digestive environment consisting of low pH and enzymes specifically designed to denature proteins.

Bacteria as prokaryotic have chemical structural components consist of macromolecules as proteins, polysaccharides and phospholipids *(16)*. All this components can easily break down by action of digestion juice, beside normal wide variation in pH in the fish digestive system. Then the foregut mucosa of fishes produces protease enzyme (e.g., pepsin) that destructed protein beside proteins are broken down in the alkaline medium of the intestine by the action of the enzyme trypsin. Fish also have enzymes which break down carbohydrates and fats (lipases) *(17)*. From this sides, using of killed bacteria as oral vaccine determined the bacterin in the shooting of the enzymes of the digestive system that remain losing of the affectivity of the vaccine from reaching to the intestinal mucosa macrophages without losing its quantity and quality. So that, artemia was used in this study as biocoater to safe the used antigen from the digestive enzymes *(18)* which documented the oral vaccine should be in formulation protect antigen from inactivation and digestion during passage through the foregut and the hindgut. The antigenic model used in this work was bacterin of *A. hydrophila.* This strain was tested for evaluation it's virulent and determination of

LD_{50} as cleared in Table 1. Based on LD_{50} of the selected *A. hyderphila* isolate was consider virulent according to classification *(19)* which reported that LD_{50} of 10^4 to 10^5 was consider in the virulent category. So, the used isolate was suitable for using as bacterin which manufactured mainly from virulent strains. The virulence degree LD_{50} was diluted to be resulting in 50% mortality.

Based on the safety test results, oral and immersion vaccinated fingerling showed mild post vaccinal reaction, while intra-peritoneum injected fingerling showed moderate skin lesion at site of inoculation. This finding supported by the results which stated that injected vaccine can cause some local inflammatory reaction at site of inoculation *(20)*.

In the (table 4) showed relative percent of survival in tilapia groups vaccinated by oral, immersion and intra-peritoneum routes with formalized whole culture vaccine of *A. hydrophila* and challenged with bath route, was 92.68%, 81.4% and 87.5% respectively. The obtained results were differ from which cited RPS for oral vaccination 63% and for immersion vaccination 89%, that mainly related to use of oral vaccination in her work without coating *(4)*.

RPS of oral vaccination was higher than that of immersion and intra-peritoneum, in addition the oral immunization route didn't cause any stress in the fish health, less labor and not damaged fish body during transferring which is predisposing factor for fungus invasion.

The humoral immune response of tilapia through micro titre agglutination test showed end points 400, 400 and 800 for oral, immersion and intra-peritoneum routes after 4 weeks and 100,100 and 200 after challenge (Table 5). Haemagglutination inhibition test showed end points 128, 128 and 256 after 4 weeks and 32, 32 and 64 after challenge (Table 6).

It has been reported that same titer of produced antibodies by oral route of immunization measured by micro titre agglutination and haemagglutination inhibition tests and also, he cited at this level oral route of vaccine administration induced protection level higher than 80% and induced significant systemic and mucosal immune response than the other routes of administration *(21)*.

The results showed closely similarity titre between oral and immersion immunized fish, these differ than that published which showed the immersion immunized is more stimulate the immune system than the oral one *(22)*. But, it has been investigated the reason at which oral route of administration induce high immune response and was explained the strategies that have been explored for improving the oral vaccination included protected antigens such as encapsulated antigens and a biofilm also, he used *A. hydrophila* for oral vaccination of carp which induced significantly higher antibody titres and protection*(12)*.

The results showed intra-peritoneum immunized fish was higher antibody titer than that recorded from oral and immersion immunized fish that may be related to the individual method of vaccine application and also, with intra-peritoneum inoculation the vaccine go in contacting directly with the immune system which documented the parental routes of antigen inoculation resulting in strong immune response *(23)*. Although intra-peritoneum immunization induces high immune response, it can't be applied on fish in large scale but, it could be applied in limited scales for fish breeders at hatcheries and even that, it cause severe stress on fish during catching and injection.

Also, the humoral immune response of tilapia under the experiment was investigated by ELISA showed optic density 1.4, 1.5 and 1.8 for oral, immersion and intra-peritoneum routes after 4 weeks and 0.4, 0.4 and 0.5 after challenge (Fig.1). ELISA results confirm that of micro titre agglutination test and haemagglutination inhibition test in assimilatory immune response between oral and immersion immunization routes. It has been recorded the same optic density of oral

and immersion immunization routes at same interval periods *(3)*.

Among the various route of immunization, the oral and immersion routes are simple, cheap and ideal for mass administration to fish of all sizes and for large scale aquaculture with possibility of quickly vaccinating large number of fish with reduced costs in addition to elimination stresses caused by parental administration.

Uncoated oral vaccination was used in a long application term reached to one week *(4)* and five days *(3)*, this is due to the bacterin was feed uncoated mixed with fish diet. This application ways cause break down of large quantity of induced antigen and back immune response resulting from the continuous applied of the antigen that leading to neutralize it from produced antibodies.

Administration of the vaccine via oral route is very attractive because it is suitable for mass administration to fish of all sizes, imposes no stress on the fish because handling is not required and therefore does not interfere with routine husbandry practices. Furthermore, oral vaccination is the only method suitable for extensive pond rearing of fish where catching the fish prior to harvest for injection or immersion vaccination is impractical. But, one of the important factors for the poor response to oral vaccination is the digestive degradation of antigens in the foregut before the vaccine reaches the immune responsive areas in the hind gut and other lymphoid organs. The encapsulation of antigen in the oral vaccination of fish were found to be a promising method due to the development of systemic induction of immune response implying that it can be applied to achieve a better protection against fish bacterial disease.

In conclusion, the current trial for fish vaccination showed that the coated vaccine when used in tilapia through the oral route was of easier administration and of higher efficacy RPS and it was effective against bacteria infection so, it can be used in diseases prevention programs.

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الملخص العربى

دراسات على التغليف الحيوى للقاح الإريموناس هيدرفيلا الميت لتحصين أصبعيات أسماك البلطى النيلى عن طريق الفم

أحمد محمد عبد الوهاب

المعمل المركزى لبحوث الثروة السمكية بالعباسة - مركز البحوث الزراعية

يتميز تحصين الأسماك عن طريق الفم بسهولة تطبيق هذا النظام حقليا حيث أنه لا يحدث أى إجهاد على الأسماك المحصنة بهذه الطريقة ويشترط لهذه الأنوع من اللقاحات أن تكون مغلفة أو محاطة ببعض التحضيرات الطبيعية أو الصناعية للحفاظ عليها من تأثير الإنزيمات الهاضمة الموجودة فى القناة الهضمية وذلك للحفاظ على التركيب الذى يسمح لها بإثارة الجهاز المناعى أثناء وجودها فى النصف الثانى للقناه الهضمية. وفى الدراسة المقدمة تم إستخدام الأرتيميا كتغليف طبيعى لتحصين ميت محضر من الإيريموناس هيدروفيلا وتم إختيار الأرتيميا لما تتميز من عدم الإخيارية الغذائية مما يجعلها تبتلع كمية كبيرة من بكتيريا اللقاح عند تحضينها معه. ثم تم إطعام أصبعيات البلطى بتلك الأرتيميا مقارنة بطريقتين أخرتين من طرق التحصين وهما عن طريق التغطيس وعن طريق الحقن.

وأظهرت النتائج أن نسب الحماية للأصبعيات بعد إختبار التحدى هى 92.68%، 81.4%، 87.5% للأصبعيات المحصنة عن طريق الفم والتغطيس والحقن على الترتيب

كما تم عمل إختبارات مناعية لمعرفة مستوى الإستجابة المناعية المتخصصة فكانت قيم إختبار التلازن المصغر هى 400، 400، 800 وقيم إختبار تثبيط التلازن الدموى هى 128، 128، 256 للأصبعيات المحصنة عن طريق الفم والتغطيس والحقن على الترتيب. كما تم عمل إختبار ELISA والذى أظهر تشابهه بين قيم المستوى المناعى للأصبعيات المحصنة عن طريق الفم والتغطيس.

والخلاصة التى تم الوصول إليها من نتائج هذه الدراسة أنه من الممكن أن يتم إستخدام لقاح مغلف بالأرتيميا عن طريق الفم فى برامج الوقاية من بكتيريا الإريموناس هيدرفيلا دون أن يتأثر هذا اللقاح بالإنزيمات الهاضمة.