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Effect of temperature, pH, and growth requirements on probiotic production isolated from dogs

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Abstract : The aim of this study was to develop specific probiotic strains of canine origin and to investigate the effect of growth requirement on its viability then microencapsulate the identified probiotic microorganisms *L. plantarum* and *L. paracasie* using skimmed milk as a wall material by the spray drying technique using inlet ant outlet temperature of 120ºC and 80ºC, respectively, drying air flow rate 85.0% of suction fan controller., The spraying system consisted of a two fluid nozzle composed of an internal tip with an opening 0.7 mm in diameter and external ring with an opening of 1.5 mm in diameter, then evaluate the tolerance of these microorganisms in the free and microencapsulated forms to pepsin (pH adjusted to 2) and oxgall bile salt 0.3 % regarding to measuring the moisture content of the two produced microencapsulated product and observing the viability and stability of probiotic bacteria during storage at 4ºC, 25ºC for 60 days for each temperature. Both micro-encapsulated microorganisms were showed high encapsulation yield reached to92.59% and 93.3 % after exposure to spray drying with moisture content of 3.4% & 3.45% for *L. paracasei* and *L. plantarum*, respectively leading to maintaining cell viability during storage till 60 days at either -4ºC & 25ºC. Also both microencapsulated microorganisms can tolerate acidic condition at pH2 and bile salt 0.3% than free cells. Trial for studying the effect of growth requirement was carried by using tryptone soya agar and showed high count for both microorganisms as it contain nutritionus substance favoured for the growth of lactobacilluis organisms.
Keywords : Probiotic, Canine, Spraydryer, Temperature, pH, Microencapsulation.

1- Introduction

Information on the canine intestinal microbiota is still extending. To adjust the disturbed microbiota and to battle infections, different therapeutic agents have been proposed, among them probiotics. In any case, available papers on probiotic applications in canine are restricted compared to data concerning humans.

The properties of probiotic microorganisms are related to host specificity[1]**.** Thus, for beneficial use as a probiotic, the bacterial species should be of host intestinal origin. Unfortunately, the available probiotics for canine commonly not originally originated from the canine GIT microbiota. In spite of that, The intestine of canine have abundant microorganisms with probiotic potential[2]**.** Beside that, studies on the quality of probiotic products for pets and their survival through the GIT is still limited. Research on the identification of novel strains and the evaluation of functional properties are being conducted[2,3,4]. As well it was proven that the original properties of probiotics may be greatly influenced by the production and manufacturing methods and the food carrier[5]. Recently, the growth media have been showed that it may significantly affect the adhesive ability of canine probiotics lactobacilli to dog mucus [6]. Also it could altered enteropathogen adhesion to canine

mucus according to the growth media used to cultivate probiotics[7]. Thus, for quality control of existing probiotics and for identification of new probiotics for pet animals the growth conditions and media should be taken in consideration, as even any change in properties may affect the outcome with different impact on host health. Beside that, the storage stability of non-viable forms of microorganisms may lead to new possibilities of obtaining nutritional supplements for pet animals.

Studies carried out have poor viability of probiotic bacteria in functional foods[8]. Protection of probotics by microencapsulation in different food carriers is one of the methods of improving their survival in functional foods.

Microencapsulation is a process which make immobilization of cells within an encapsulating material[9].

It was demonstrated that spray-drying among microencapsulation techniques has advantages of low operating cost with low moisture content for large-scale production of powdered microbial cultures[10,11], better storage stability than frozen or fresh cultures[12,13], better survival, and functionality when subjected to simulated gastrointestinal conditions[14,15], and the possibility of storage at room temperature[16,17,18].

Several food carriers have been employed for the encapsulation of probiotics by spray-drying, such as starches[19,20,21], maltodextrin[14,22], gums[13,17,23], gelatin[22], whey proteins[21,23,24], sweet whey[15], and reconstituted skim milk[25,26]. Skimmed milk was proved to be the most effective material for keeping up cell viability during spray- drying process [27,28,29,30]. Several authors suggest that the efficiency of the dairy matrix in maintaining cell viability during drying is attributed to the components of milk [16,27]. During water removal, lactose is thought to interact with the polar head groups of phospholipids and proteins of the cell membrane, thus reducing damage to the membrane during spray-drying and enhanced storage[16,31,32,33]. Microencapsulation has been proposed to enhance the survival of probiotic strains during passage through gastrointestinal tract[24,30,34]. It is also helpful in evaluating survival of probiotics in different food matrices during passage through the gastrointestinal tract [30,35,36]. In addition to the food matrix, the probiotic's resistance to the gastrointestinal conditions depends on intrinsic properties of each strain and various testing conditions, such as pH, presence of enzymes, temperature and time of incubation[37,38,39,40,41]. The aim of this study was to develop species specific probiotic of canine origin and to investigate the effect of growth requirement on its viability, then microencapsulate the obtained probiotic using skimmed milk as a wall material applying the spray drying technique using inlet and outlet temperature of 120ºC and 80ºC (±2.0ºC), respectively , drying air flow rate 85.0% of suction fan controller, and to evaluate the tolerance of these microorganisms in the free and microencapsulated forms to pepsin (pH adjusted to 2) and oxgall bile salt 0.3 % regarding to measuring the moisture content of the two produced microencapsulated product and observing the viability and stability of probiotic bacteria during storage at -4ºC, 25ºC for 60 days for each temperature.

2- Materials and methods

Fresh fecal swabs were collected from 16 healthy dogs, swabs were cultured directly on de Man, Rogosa, and Sharpe (MRS) agar (oxoid) incubated anerobically at 37 ºC for 18-24 hour.

All isolates obtained were characterized as lactobacilli based on Grams stain, biochemical fermentation tests and catalse test. The final confirmation of their identity was achieved by using vitek 2 compact apparatus. 11 isolates were identified as *lactobacilluis plantarum* and the other 5 isolates identified as *lactobacilluis paracasei.*

Cultures were maintained by continuous subculturing on MRS agar plates and inoculation in MRS broth containing 15% glycerol and stored at -80ºC.

Studying the effect of growth requirement for Lactobacilluis strains[7]

The two selected strains were grown in de man, rogosa, and sharpe agar (MRS; oxiod) at 37ºC anerobically for 18-24 h. For a comparison of growth requirement a parallel assay was preformed where strains were cultured in Tryptone soya agar contain soya meal as a source of nitrogen for lactic acid producing bacteria. Both cultures were inoculated into MRS broth and tryptone soya broth anerobically at 37ºC for 18-24 h then

serial dilutions were obtained by adding 900 micron of peptone saline to 100 micron of bacterial suspension to compare between the total colony count on each media.

Probiotic Culture Preparation for encapsulation [42]

For preparing the probiotic cell suspension for encapsulation process reactivation of frozen culture occurred twice in de Man, Rogosa, and Sharpe (MRS) broth (1% vol/vol) and incubated at 37ºC for15 h. then centrifugation of cell suspension at 3000 rpm for 10 min at 4ºC.washing the concentrated cells that obtained after centrifugation twice with sterile peptone solution (0.1%wt/vol), with continuous disposal of the supernatant and centrifugation as previously described. After the second wash, the cell concentrate was resuspended into 0.1% peptone solution.

Production and Storage of Probiotic Microparticles[42] **with modification:**

Solution with 30% total solid (500 L) was prepared from skimmed milk powder (LAB-M) reconstituted in sterile water (121ºC for 15 min) at room temperature The solution were maintained under stirring at 450 rpm for 30 min to improve reconstitution of the powder. After that 1% (vol/vol) of each strain were added to the solution separately then ,reconstituted skimmed milk was atomized for drying and microencapsulation of both strains using spray dryer (A co-current Mini Spray Dryer B-290 (BÜCHI, Flawil, Switzerland) using inlet and outlet temperature of 120ºC, 80ºC (±2.0ºC), respectively , drying air flow rate 85.0% of suction fan controller., The spraying system consisted of a two-fluid nozzle composed of an internal tip with an opening 0.7 mm in diameter and an external ring with an opening of 1.5 mm in diameter. After spray drying, two products were obtained skimmed milk microparticles containing *lactobacilluis plantarum* and skimmed milk microparticles containing *lactobacilluis paracasei*. Five gram of each product were packed, and stored at 4ºC and 25ºC for 60 days.

Counting of *L. plantarum and L. paracasei* and their encapsulation yield[42]

The survival of *L. plantarum and L. paracasei* were detected by counting the viable cells in both the encapsulating material after inoculation and in the microparticles immediately after production (time zero), 7, 15, 30, 45 days of storage at 4ºC and 25ºC. One milliliter of encapsulating material was diluted into 9 ml of saline peptone solution (pH 7.2). To evaluate the viability of microencapsulated probiotic, 1 g of each sample was resuspended in 9 ml of saline peptone solution, stirred by vortexing for 1 min, and allowed to stand at room temperature (~25°C) for 20 min to reconstitute the powder. Serial dilutions were performed in saline peptone solution followed by pour plating on MRS agar and incubation at 37°C for 18-24 h in anaerobic condition.

The survival of *L. plantarum* and *L. paracasei* was determined by counting the colony-forming units. The encapsulation yield (EY) was calculated according to [14] with modifications by [42]**,** and is presented in the following equation.

$$EY = (\log N/\log N0) \times 100$$

Where N is the number of viable cells (cfu/g of DM) in the powder and N0 is the number of viable cells (cfu/g of DM) in the encapsulating material before drying.

Effect of acid and bile:

Simulated gastric juice (SGJ) was prepared according to the method of[43] to determine the survival of encapsulated probiotics in simulated acidic condition. MRS broth was adjusted to pH 2.0 with 1 MHCl and sterilized by autoclaving at 121°C for 15 min. Pepsin (3,000 µ/g) was filtered through a 0.22 µm sterile membrane filter then added to sterile MRS broth for a final concentration of 0.3 % (v/v). Simulated intestineal juice (SIJ) was prepared by dissolving bile salt in MRS broth to a final concentration of 2 % (w/w) and sterilized by autoclaving at 121°C for 15 min. Microcapsules (0.5 g) were added to 9.5 g of sterile SGJ or SIJ and incubated at 37°C for 3 h at constant agitation (100 rpm). The samples were taken at 0, 1, 2, and 3 hours, and the relative cell viability (a ratio of viable cells at time 't' to that at time zero h) was evaluated in simulated acidic or bile conditions.

Moisture content [43]

The average moisture content (wet basis) of SD powder was measured. A known mass of sample (0.5 g) was placed in aluminium foil pan and dried in a vacuum oven at 100ºC for a period of 12 h. The initial and final weights were used to calculate the wet basis moisture contents. The experiments were carried out in duplicates and averaged values were taken to calculate.

3- Results and Discussion:

Table (1): Comparative count of *L. paracasei* and *L. plantarum* using tryptone soya agar and MRS:-

MRS	Tryptone soya agar
$5x\ 10^7$	$2x\ 10^7$
$7x\ 10^7$	$6x\ 10^7$

Results in Table (1) shows high growth rate when *L. paracasei* and *L. plantarum* cultivated in tryptone soya agar approximately near that in MRS where Tryptone Soya Agar contains enzymatic digests of casein and soybean meal, which serves amino acids and other nitrogenous substances which support the growth of lactobacilluis microorganisms. Other authors used whey supplemented with yeast extract for inoculation of *L. reuteri* showing increase in bacterial population by 5 log cycles **(44).**

Table (2): Survival of *L. paracasei* in pepsin (PH2) in free and microencapsulated form

Time in hour	0	1	2	3
Free	9.8×10^8	8.4×10^8	6.8×10^7	5.8×10^7
microencapsulated	7.5×10^8	7.8×10^8	8.1×10^8	8.3×10^8

Table (3): survival of *L. plantarum* in pepsin in free and microencapsulated form

Time in hour	**0**	**1**	**2**	**3**
Free	7.5×10^{10}	5.4×10^{10}	3.5×10^8	3.1×10^8
microencapsulated	7.0×10^{10}	7.3×10^{10}	7.35×10^{10}	7.5×10^{10}

Table (2) and (3) show the viability of free and microencapsulated *L. paracasei* and *L. plantarum* respectively when subjected to simulated gastric conditions. In case of *L. paracasei* After 1 hour of exposure to gastric condition ,there is no significant reduction in count for free probiotic culture and reduced by 1 log cycle after 2 hours (6.8×10^7 cfu/ml) and finally after 3 hours of exposure the count reached to 5.8×10^7 cfu/ml where initial count was 9.8×10^8 cfu/ml, on the other hand microencapsulated *L. paracasei* in skimmed milk after 1 hour of exposure can maintain its count with slight increase and after 3 hours reaching to 8.3×10^8 as started with 7.5×10^8. In case of *L. plantarum* free cells showed little resistance to PH2 as initial concentration was 7.5×10^{10} and the count decreased gradually till reached after 3 hours to 3.1×10^8 while after microcapsulation the initial count was 7.0×10^{10} and can tolerate PH2 as count increased to 7.5×10^{10} cuf/ml after 3 hours Thus, this result showed that the survival of both microcapsulated *L. paracasei* and *L. plantarum* was significally higher than the free one when subjected to gastric condition at pH 2 which confirmed the protective effect of skimmed milk as encapsulating material which is demonstrated by other authors as *Lactobacillus plantarum* CFR 2158100 maintained 100% survival when microencapsulated by spray-drying using skim milk and subjected to pH 2.0 for 4 h [14], whereas others studying other encapsulating material as ALG for coating *L. paracasei* the free organisms showed a constant reduction in viability when exposed to acidic conditions. Initially, free cells had an average count of 9.69 log cfu/ml of viable probiotic bacteria, but after 1 h of exposure the population of cells was decreased to 6.39 log cfu/ml and finally reached to 2.71 log

cfu/ml after 2 h of exposure, however in ALG microcapsules after 1 hour of exposure the probiotic viability was 7.1 log cfu/ml and after 2 h of exposure was 5.41 log cfu/ml [9].

Table (4): Comparative viability of *L. paracasei* and *L. plantarum* in free and microcapsulated form to bile:-

	0hr	**1hr**	**2hr**	**3hr**
Free *L. Paracasei*	$8.4x10^8$	$6.4 x10^8$	$4.5 x10^7$	$3.5 x10^7$
Microcapsulated *L. Paracasei*	$7.5 x10^8$	$7.8 x10^8$	$8.5x10^8$	$8.7x10^8$
Free *L. Plantarum*	$6x10^{10}$	$3.2 x10^{10}$	$1.6 x10^8$	$1.1 x10^8$
Microcapsulated *L. plantarum*	$7.0 x10^{10}$	$7.6 x10^{10}$	$7.9 x10^{10}$	$8.5 x10^{10}$

Results in table (4) showed that both *L. paracasei* and *L. plantarum* can withstand bile salt in free form as after 2 hours exposure to bile decreased by one log cycle in case of *L. paracasei* and 2 log cycle in case of *L. plantarum* but still within limit required to be found in dietary formula on the other hand the encapsulated product can maintain the count of probiotic strains with observed increase among 3 hours of incubation with bile salt as started with $7.5 x 10^8$/ml and ended with $8.7x 10^8$/ml for *L. paracasei* and $7.0 x 10^{10}$ ended with $8.5 x 10^{10}$ cfu/ml for *L. plantarum* these results could be related to the effect of gastric condition on digestion of casein producing amino acids and peptides which enhance the growth of probiotic bacteria [45,46] and liberation outside the capsule when reaching intestinal phase at neutral PH [24].

Table (5): Effect of spray drying on survival of *L. paracasei* and *L. plantarum* and their moisture content

	Before spray drying	**After spray drying**	**Encapsulation yield (EY)**	**Moisture content %**
L. paracasei	$8.1 x10^8$	$7.5 x10^8$	92.59%	3.5%
L. plantarum	$7.5 x10^{10}$	$7.0 x10^{10}$	93.3%	3.45%

Table (5) shows the viability of probiotic microcpsulated organism before and after subjecting to spray drying process and their encapsulation yield.

Several authors suggested that the influence of dairy carriers in maintaining cell viability during drying is correlated to the presence of lactose and milk proteins, as these proteins may interact with the cell membrane of the microorganisms, inhibiting the leakage of the membrane during water removal[28]. The physical state of cell membrane is converted from liquid crystalline phase to leak gel phase due to removal of water bound to phospholipids[32]. During drying process, the interaction between the cell membrane phospholipids and mainly lactose enhance delaying membrane phase conversion, preventing its leakage and improving bacterial recovery after rehydration[16,31,32,33]. In this study the encapsulation yield is high in both microencapsulated probiotic cells as 92.59%, 93.3% for *L. paracasei* and *L. plantarum*, respectively which is related to composition of skimmed milk as it contain proteins mainly casein and lactose which protect cell during drying[47].

Inlet /outlet temperature was proved to be Another factor that effect the survival of probiotic cells during spray drying process as the lowest inlet/outlet temperature the least leathel effect on probiotic cells here our result agree with [37] as the count of *B. lactis*, revealed no change , going from $1.8 x 10^8$ cfu/g at the start of the process to $1.3 x 10^8$ cfu/g at the end of applying inlet /outlet temperature 130ºC and 75ºC, respectively. On the other hand Moisture content decreased by increasing drying temperature. Here our data revealed moisture content of 3.5 % and 3.45% for *L. paracasei* and *L. plantarum*, respectively as they subjected to the same condition for drying process this percent is within the limit required for protecting cell damage during storage of dried cultures[13,17]. As it must not exceed 4% for skimmed milk[48].

Table (6): Viability of *L. paracasei* during storage

	7 days	**15 days**	**21 days**	**30 days**	**45 days**	**60 days**
4ºC	$5.5 x10^8$	$5.32 x10^8$	$4.75 x10^8$	$4.6 x10^7$	$3.5 x10^7$	$3.2x10^7$
25ºC	$5.2 x10^8$	$4.9 x10^8$	$4 x10^8$	$3.5x10^7$	$2.3x10^7$	$2x10^7$

Table (7): Viability of *L. plantarum* during storage

	7 days	**15 days**	**21 days**	**30 days**	**45 days**	**60 days**
4ºC	6.8 $x10^{10}$	5 $x10^{10}$	4.7 $x10^{10}$	8 $x10^{8}$	7.4 $x10^{8}$	6 $x10^{8}$
25ºC	4.5 $x10^{10}$	4 $x10^{10}$	3.3 $x10^{10}$	7 $x10^{8}$	5 $x10^{8}$	4.3 $x10^{8}$

Determination the shelf-life time of the probiotic microparticles after production is necessary as it is not subjecting to damage during processing only but also it may undergo cell damage during storage .here data showed that storage at 4ºC is slightly higher than that at 25 ºC for both microparticles as the metabolic rate of probiotic bacteria increased at room temperature which resulted in accumulation of toxic product and consequently reduce viability of bacteria[49]**.** Population of probiotic cell can withstand storage condition till 60 days it reaches to 3.2 $x10^{7}$, 6 $x10^{8}$ for *L. paracasei* and *L. plantarum*,respectively at 4ºC, and reduced by 2 log cfu/g at 25ºC where it became2$x10^{7}$ and 4.3 $x10^{8}$ for *L. paracasei* and *L. plantarum*, respectively, overall the count for both temperature is higher than 10^{6} log cfu/g which is within limit required to be found in food to exert its probiotic benefits [50,51]**.** These results may be due to encapsulating material, low moisture content of microparticles, main characteristic of probiotic strain[17,52,53]**.**

4- Conclusion

Microencapsulation of *L. paracasei* and *L. plantarum* in skimmed milk using spray dryer showed a good potential for use as probiotic for canine as skimmed milk can maintain cell viability during passage in acidic pH (2) using pepsin and can tolerate oxgall bile salt 0.3% for 3 hours comparing with free *L. paracasei* and *L. plantarum* also microencapsulated organisms can withstand the effect of temperature during the process of drying using inlet/outlet temperature 120ºC /80ºC resulting in producing microencapsulated probiotic with low moisture content not exceed 4% which is within limit required for probiotic to exsert its beneficial effect. Behind that microcapsulation can maintain viability of probiotic cell till 60 days at 4ºC and 25ºC .Also it was found that tryptone soya agar is suitable for the growth of lactobacilluis microorganisms and showed high count near to that obtained by MRS.

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