

Adhesion of Probiotic Bacteria to Resistant Rice Starch

Ghalia Salem, Zaiton Hassan and Maryam Abubakr

Department of Science and Technology,
University Sains Islam Malaysia (USIM), Bandar Baru Nilai, 71800 Nilai Negeri Sembilan, Malaysia

Received 2012-07-27, Revised 2012-08-26; Accepted 2013-04-25

ABSTRACT

Resistant starch as prebiotics can be combined with probiotics to increase their survival during processing of food products. In present study Lactic Acid Bacteria (LAB) which, isolated from (yoghurt, banana and human breast milk) were screened for their probiotic properties and evaluated for the adhesion properties to Rice Resistance Starch (RRS). 26% of the isolates (10/39) overcame the stress to pH 3 and 0.3% bile indicating their probiotic properties. All ten LAB isolates adhered to RRS within 60 min of exposure. Isolates Bn1 and HM2 were highly adhered to RRS with a total of 79 and 77% of the cells adhering, respectively. Moderate adherent was observed by isolates FY (55%), YN (70%), CY (48%), HM1 (61, 5%), HM3 (65%) and HM4 (50, 5%), while isolate YD and Bn2 were poorly adhered to RRS (<40% adherent). NaCl and Tween 80 did not influence on adhesion capacity but, the adhesion was inhibited by protease and by low pH. Different effect on adhesion of probiotic bacteria to resistant rice starch was caused by monosaccharides, disaccharides and polysaccharides, depending on their molecular size. The effect of gastric condition (in vitro) appeared, inhibition in adhesion by protease and low (pH<3). While bile has not affected on adhesion, but the pancreatin caused weakening binding capacity of the starch. It might be possible to utilization adhesion of probiotic and prebiotic in microencapsulation and synbiotic food applications such as bakery products.

Keywords: Resistant Starch, Lactic Acid Bacteria, Prebiotic

1. INTRODUCTION

Probiotics are live microorganisms that are created food supplements order to promote health for costumers by adjust microbial balance in the intestine (Kosin and Rakshit, 2006). Since a wide circulation of *Lactobacillus* and *Bifidobacteria* species in the intestinal tract is generally considered to be signal of a healthy microbiota, they become commonest probiotic bacteria used for a variety of health problems. Loosing probiotic during processing is the most important technological obstacle that must to be solved. Produce large enough amounts of viable and stable probiotic cultures has been technological challenge for industry. Some studies demonstrated that high levels of viable probiotics are recommended in probiotic foods to be functional (Galdeano and Perdigon, 2004).

Prebiotics like resistant starch can be combined with probiotics as synbiotic to make system more resistant to surrounding conditions and more effective (Kosin and Rakshit, 2006). Resistant starch is the starch that can escape from digestion the small intestine by amylases enzyme and arrived to colon where it will be fermented without loosing (Sajilata *et al.*, 2006; Anal and Singh, 2007). This system provides protective device of probiotics is reliable in media simulating the gastric fluid and then probiotics are released in the large intestine. while, resistant starch can be used by probiotics in the large intestine as energy source (Mortazavian *et al.*, 2008).

Adhesion of different species of probiotics such as *Bifidobacterium* spp to native maize, oat, potato and barley starch granule showed that are not all the *Bifidobacteria* strains have adhesive characteristic to

Corresponding Author: Ghalia Salem, Department of Science and Technology, University Sains Islam Malaysia (USIM), Bandar Baru Nilai, 71800 Nilai Negeri Sembilan, Malaysia

starch granules (Crittenden *et al.*, 2001) furthermore, the researchers are not sure about if there are any effect by adhesion in starch metabolism by probiotic. The present work studied the ability of LAB isolated from different sources with potential probiotic properties to adhere to several resistant rice starch granules, to achieve a preliminary comprehension of the adhesion mechanisms.

Probiotic adhesion to starch may add advantages in new probiotic technologies through protection these probiotics during processing and storage, also to target the release of the bioactive to large intestinal tract. The researchers lately developed microencapsulation technology by addition of Prebiotic compounds, such as starch to promotes the survival of Probiotic bacteria. This technique is used to ensure protection probiotic bacteria from untoward environmental conditions where they may not normally survive. Thus, other aim of this study was to estimate The possibility of utilization from adhesion of probiotic and prebiotic in microencapsulation and synbiotic food applications such as bakery products.

2. MATERIALS AND METHODS

2.1. Starch Isolation from Rice

According to Wang and Wang (2001) method, 100 g rice flour was soaked in 200 mL NaOH at concentration of 0.1% for 18 h. The slurry was mixed robustly by a blender (BERJAYA 1/BSP-CB2L) for 2 min. then passing the slurry through screen (63 μ m) and centrifuged at 1400 \times g for 10 min. The residue was washed with 0.1% NaOH and water, then PH was adjusted to pH 6.5 by Hcl (1.0 N), then centrifuged again. lastly The starch was washed three times by deionized water then dried at an oven(45 $^{\circ}$ C, 48 h).

2.2. Resistant Rice Starch (RRS) Formation

Resistant rice starch was prepared following the method of Pongjanta *et al.* (2008), as every 15 g of rice starch was mixed with 100 mL of distilled water (15%w/v). The mixtures were kept at 30 $^{\circ}$ C for 1 h with subjected them to strong shake by occasional vigorous shaking. Then samples were heated to approximately 75 $^{\circ}$ C for 30 min. The gelatinized samples were cooled to 55 $^{\circ}$ C, which was ideal temperature for pullulanase enzyme. 8 unit pullulanase enzymes per gram starch were added at 55 $^{\circ}$ C for 48 h in water bath. When the enzyme treatments were completed, the samples were autoclaved at 121 $^{\circ}$ C for 15 min. The samples were incubated at 4 $^{\circ}$ C for 16 h then subjected to one cycle of freezing and thawing process (-10/30 $^{\circ}$ C). The resistant starch was collected and dried at an oven (45 $^{\circ}$ C). Then the samples were milled and sieved through a 100- mesh sieve and packaging away from moisture at room temperature for further determinations.

2.3. Isolation of Lactic Acid Bacteria from Different Sources

2.3.1. Yoghurt and Fermented Banana

Locally available fermented dairy products, namely: Yoghurt Natural (YN), Children Yoghurt (CY), Yoghurt Drink (YD), Fruit Yoghurt (FY) and Fermented banana, which all claimed to contain Lactic Acid Bacteria (LAB), was used in this study as sources of LAB. Three (3) samples of each product were bought from the several supermarkets were obtained. About 10 g of sample was added to 90 mL 0.1% peptone water and appropriate dilution was spread plated on de Man, Rogosa and Sharpe (MRS) agar (Oxoid CM0361) plates containing 0.8% calcium carbonate (CaCO₃). Plates were incubated anaerobically (in anaerobe jar using Oxoid anaerogen compact) at 37 $^{\circ}$ C for 48 h. The bacterial strains were kept at -20 $^{\circ}$ C after adding 15% glycerol stock. Each bacterial strain was sub-cultured at least three times (1%, v/v) at 24 h before using in subsequent study (Kheadr, 2006).

2.4. Human Breast Milk

The samples were collected sterilely from 10 mothers were in a good health then delivered to the laboratory on ice. Samples were diluted to 10⁻¹, 10⁻² and 10⁻³ using sterile peptone water, then 1ml of each sample were poured into MRS-cystein agar (pH 5.5). The plates were incubated Anaerobically at 37 $^{\circ}$ C for 3 days (Yavuzdurmaz, 2007).

2.5. Probiotic Properties of Isolates

2.5.1. Oxbile Tolerance

The tolerance of LAB strains to oxbile was evaluated by following Kheadr (2006) method. The mixture of MRS broth (Oxoid CM0359) with 0.3% w/v oxbile was prepared then filled each well of sterile flat-bottom 96-well microtiter plate (Falcon, Becton Dickinson and Company, Franklin Lakes, NJ, USA) by 150 μ L and 30 μ L of the overnight culture which diluted to 1/1000 in the same broth. Then incubated the Microplate at 37 $^{\circ}$ C for 24 h under anaerobic conditions. A biophotometer (Eppendorf Asia Pacific Sdn. Bhd) at 600 nm was used to measured the optical densities.

2.6. Acid Challenge

Ten mL of cultures which incubation with MRS until reached mid-log-phase were obtained by centrifugation at 6,000 \times g for 15 min at 4 $^{\circ}$ C (AllaegraTM 25R centrifuge, Beckman Coulter TM), then resuspended in the same volume of MRS broth (Oxoid CM0359) at pH 2.0. Then incubated the suspension at 37 $^{\circ}$ C for 60 min under anaerobic conditions.

Table 1. Treatments used in the investigation of mechanisms of adhesion

Treatment	Description
Phosphate buffer	The adhesion experiment was performed in 0.1 M phosphate buffer (pH 7.0)
Fresh medium	The adhesion experiment was performed in fresh growth medium (pH 6.8)
Spent medium	The adhesion experiment was performed in spent supernatant from a 24-h culture of the bacterial strain (pH 4.5)
Pepsin-treated spent medium	The adhesion experiment was performed in spent medium treated with 30 U of pepsin A (EC 3.4.23.1; Sigma Chemical Co., St. Louis, Mo.) mL^{-1} for 6 h at 37°C; for pepsin treatment, the pH of the medium was reduced to 2.0 by using 0.1 M HCl and then readjusted prior to the adhesion experiment to 4.5 by using 0.1 M NaOH
Pepsin-treated starch	The adhesion experiment was performed by using RRS that had been treated with 30 U of pepsin mL^{-1} (at pH 2.0) for 6 h at 37°C; RRS was washed and resuspended in phosphate buffer (pH 7.0) for the adhesion experiment
ProteinaseK-treated cells	Cells (10^8 CFU mL^{-1}) were pretreated with 10 U of proteinase K (Roche Molecular Biochemicals, Basel, Switzerland) mL^{-1} at pH 7.0 for 6 h at 37°C; the cells were then washed twice and the adhesion experiment was performed in 0.1 M phosphate buffer (pH 7.0)
NaCl	The adhesion experiment was performed in 0.1 M phosphate buffer (pH 7.0) containing 0.5 M NaCl
Tween 80	The adhesion experiment was performed in 0.1 M phosphate buffer (pH 7.0) containing 3.0 g of Tween 80 L^{-1}
Pepsin-treated cells	Cells (10^8 CFU mL^{-1}) were pretreated with 30 U of pepsin mL^{-1} at pH 2.0 for 6 h at 30°C; the cells were then washed twice and the adhesion experiment was performed in 0.1 M phosphate buffer (pH 7.0)
Effect of potential inhibitors	The adhesion experiment was performed in 0.1 M phosphate buffer (pH 7.0) containing 5 g of one of the following carbohydrates per liter: glucose, maltose, lactose, sucrose, maltodextrin and amylose

Source: Crittenden *et al.* (2001)

To determination viability of bacteria before and after incubation 10 g of sample was added to 90 mL 0.1% peptone water and appropriate dilution was spread plated on de Man, Rogosa and Sharpe (MRS) agar. Plates then incubated anaerobically at 37°C for 48 h (Kheadr, 2006).

2.7. Oxbile Challenge

Ten mL of cultures which incubation with MRS until reached mid-log-phase were obtained by centrifugation at $6,000 \times g$ for 15 min at 4°C, then resuspended in the same volume of MRS broth at pH 6.5 containing 0.3% (w/v) oxbile. Then incubated the suspension at 37°C for 90 min under anaerobic conditions.

To determination viability of bacteria before and after incubation 10 g of sample was added to 90 mL 0.1% peptone water and appropriate dilution was spread plated on (MRS) agar. Plates were incubated anaerobically at 37°C for 48 h (Kheadr, 2006).

2.8. Determination of Adhesion Level by a Co-Sedimentation Assay

Determination of adhesion level by co-sedimentation assay followed the method of Crittenden *et al.* (2001). The cells were washed twice with 10 ml of 0.1 M phosphate buffer (pH 7.0), then they are suspended in the same buffer (concentration = 10^7 cells mL^{-1}). In a 1-cm-diameter test tube two milliliters of the bacterial suspension were mixed with an equal volume of a suspension of starch granules (10 g L^{-1}) in 0.1 M phosphate buffer (pH 7.0). The suspension was let for 1 h at room temperature to allow the starch to sediment. Two 1.5 mL samples were then taken from 0.5 cm below the liquid surface and by a spectrophotometer the optical density was measured (at 540 nm). Then find the result as follows:

$$\text{Percentage of cells adhering to starch} = a - b/c$$

a = OD540 of a sample containing starch plus bacteria

b = OD540 of a sample containing starch but no bacteria

c = OD540 of a sample containing bacteria but no starch

Highly adherent (more than 70% of the cells adhered to the starch) as (40-70% adhesion) were named as moderate however less than 40% adhesion were named as poor adherent strains.

Table 2. Treatments used to simulate conditions for adhesion during passage through the upper gastrointestinal tract

Treatment	Description
Effect of pH	The adhesion experiments were performed in 0.1M citrate-phosphate buffers at pH 2.0, 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0
Effect of acid plus pepsin	The adhesion experiments were performed in 0.2 M HCl-KCl buffer (pH 3.0) containing
Effect of bile	30 U of pepsin A (EC 3.4.23.1; (Sigma Chemical Co.) m L ⁻¹ The adhesion experiments were performed in 0.1 M phosphate buffer (pH 7.0) containing 3.0 g of bile (Sigma Chemical Co.) L ⁻¹
Effect of pancreatic	RRS was pretreated for 6 h at 37°C with 0.01 g of pancreatic (Sigma Chemical Co.) g ⁻¹ ; the RRS was washed twice and the adhesion experiments were performed in 0.1 M phosphate buffer (pH 7.0)

Source: Crittenden *et al.* (2001)

2.9. Study of Adhesion Mechanisms

Studies of the mechanisms of adhesion were aimed to determine whether adhesion was due to the growth medium components, hydrophobic or electrostatic interactions, or specific cellular or extracellular proteins produced by the bacteria. In addition, to determined nature of the receptor sites in the adhesion reaction, study the effect of some inhibitors of adhesion inclusive: glucose, maltose, sucrose, lactose, amylose and maltodextrin was done. The treatments used in the adhesion assay are described in (Table 1). The experiments were performed with two adherent strains Bn1 and Hm2. Each experiment was performed three times. The bacteria were in the stationary phase when they were used in the adhesion assay. For each assay, 2 mL of bacterial suspension were mixed with same volume of starch (10 g L⁻¹) suspended in the appropriate treatment medium (phosphate buffer, fresh medium).

2.10. Adhesion Under Conditions that Simulated the Upper Gastrointestinal Tract Conditions

To determine the effect of some of the conditions of the stomach and small intestine on bacterial adhesion to RRS, adhesion experiments were performed in vitro under conditions simulating the physiological conditions in the upper gastrointestinal tract (Table 2).

2.11. Statistical Analysis

The results are presented as mean \pm standard deviations of three replicate determinations and were statistically analyzed by two-way Analysis of Variance (ANOVA) at ($p \leq 0.05$).

3. RESULTS

3.1. Isolation of LAB

About 39 bacteria were isolated from different sources. From those isolates 18 showed clear zone on

modified MRS-CaCO₃ agar, catalase test negative and Gram positive and were considered as LAB (Table 3).

3.2. Probiotic Properties of Isolates

3.2.1. Oxbile Tolerance

Previous studies proved That bile concentration of the human gastro intestinal tract is 0.3% w/v. Growth was monitored at OD600, it was observed that all ten isolates showed varied degree of growth when grown on MRS medium supplemented with concentration (0.3%) of bile salt. From Table 4, it was observed that HM2 showed the highest value of OD600 (1.23) while Bn2 showed the lowest absorbance (0.689). Other isolates were in the range of 0.711 to 1.092.

3.3. Acid and Oxbile Challenge

Survival of isolates after acid and oxbile stresses is shown in (Table 5). Showed viable counts (log¹⁰ cfu/mL) of probiotic isolates at the beginning and end of acid and oxbile stress and percentage survival of the isolated strains in the acid and oxbile stress (Table 6). All ten isolates could survive and increased viability in acidic condition by 0.01 to 0.35 Log¹⁰ cfu/mL.

3.4. Determination of Adhesion Level by a Co-Sedimentation Assay

Ten species of bacteria adhered to rice starch granules with in 60 min of exposure to the granules (Fig. 1). Bn1 and HM2 adhered well to rice starch with a total of 79 and 77% of the cells adhering (highly adherent). YN = 70, CY = 48, FY = 55, HM1 = 61, 5, HM3 = 65 and HM4 = 50, 5% species adhered less well (moderate adherent). YD = 20 and Bn2 = 18% species adhered less than 40% (Poorly adherent).

Table 3. Phenotypic characteristics of LAB isolated

No.	Source	Code	CaCO ₃ ^a	Catalase reaction	Gram reaction	Gas from glucose	Cell morphology
1	Yoghurt natural	YN1	+	-	+	-	Rod
		YN2	+	-	+	+	Coccid
		YN3	+	-	+	+	Coccid
2	Children yoghurt	CY	+	-	+	-	Rod
		YD1	+	-	+	-	Short rod
3	Yoghurt drink	YD2	+	-	+	+	Rod
		FY1	+	-	+	-	Rod
4	Fruit yoghurt	FY2	+	-	+	-	Rod
		Bn1	+	-	+	-	Short rod
5	Banana	Bn2	+	-	+	-	Rod
		Bn3	+	-	+	+	Coccid
		Bn4	+	-	+	+	Coccid
		Bn5	+	-	+	+	Rod
		HM1	+	-	+	+	Short rod
6	Human milk	HM2	+	-	+	+	Short rod
		HM3	+	-	+	+	Rod
		HM4	+	-	+	+	Rod
		HM5	+	-	+	-	Coccid

(+) positive, (-) negative

Table 4. Growth of LAB in 0.3% oxbile^a

Isolates	OD600
YN	0.961
CY	0.711
YD	1.092
FY	0.933
Bn1	1.004
Bn2	0.689
HM1	0.777
HM2	1.230
HM3	0.867
HM4	0.822

A Titer plates were incubated at 37°C for 24 h anaerobically and growth was monitored at OD600 nm

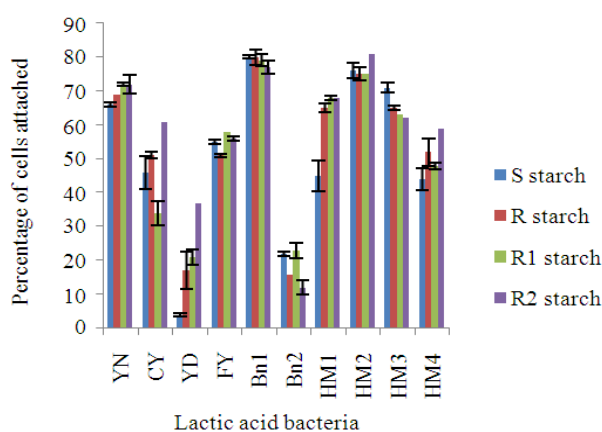


Fig. 1. Percentage of cells adhering to resistant rice starch (S = soluble starch, R = white rice starch, R1=unpolished brown rice starch, R2 = grow Cambodian brown rice starch). Error bars indicate ±1 standard deviation from the mean (n = 3)

Table 5. Viable counts (log₁₀ cfu/mL) of LAB isolates at the beginning and end of acid and oxbile stress experiments

Isolates	Acid stress ^a		Oxbile stress ^b	
	0 min	60 min	0 min	60 min
YN	9.08±0.06	9.11±0.03	9.29±0.03	9.34±0.01
CY	8.26±0.15	8.29±0.25	8.81±0.21	9.17±0.08
YD	8.98±0.05	9.23±0.16	9.18±0.09	9.27±0.03
FY	8.55±0.08	8.56±0.10	8.84±0.01	9.11±0.06
Bn1	9.12±0.00	9.31±0.06	9.00±0.00	9.17±0.03
Bn2	7.00±0.29	7.35±0.00	7.03±0.00	7.47±0.19
HM1	7.60±0.46	7.69±0.46	7.40±0.32	8.26±0.15
HM2	9.70±0.02	9.92±0.02	8.77±0.04	9.37±0.05
HM3	8.90±0.19	8.98±0.09	9.00±0.26	9.33±0.06
HM4	7.11±0.26	7.20±0.46	8.34±0.28	8.37±0.23

(a) Cells kept for 60 min in MRS broth adjusted to pH 2.0 at 37°C (b) Cells exposed to 0.3% (w/v) oxbile in MRS broth at pH 6.5 at 37°C

Table 6. Percentage survival of the LAB isolates strains in the acid and oxbile stress

Isolates	Survival (%)	
	Acid stress ^a	Acid stress ^a
YN	3	5
CY	3	36
YD	25	9
FY	1	27
Bn1	9	17
Bn2	35	44
HM1	9	86
HM2	22	60
HM3	8	33
HM4	9	3

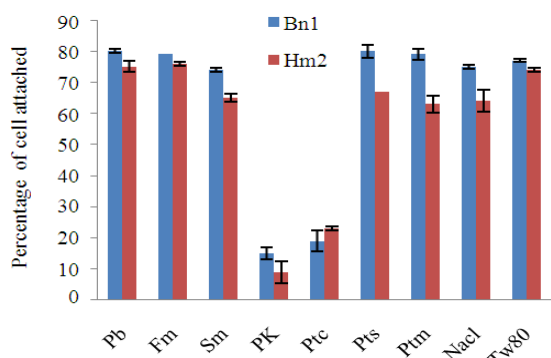


Fig. 2. The effect of chemicals (NaCl, Tween 80 and Pho phosphate buffer), pepsin and proteinase (pts pepsin-treated starch, ptc pepsin-treated cells, pk protenase-K-treated cells) on the attachment of probiotics to resistant rice starch. Error bars indicate ± 1 standard deviation from the mean (n=3)

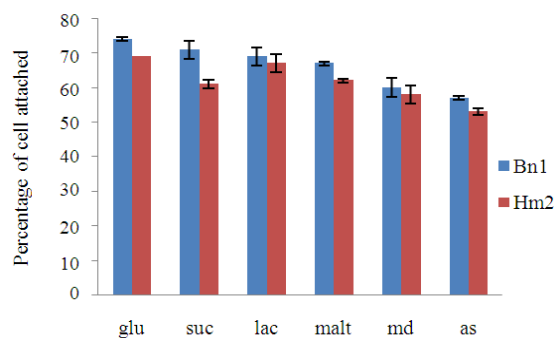


Fig. 3. The effect of monosaccharides (glu glucose) disaccharides (suc sucrose, lac lactose, malt maltose) polysaccharides (md maltodextrin, as amylose) on the adhesion of probiotics to resistant rice starch. Error bars indicate ± 1 standard deviation from the mean (n=3)

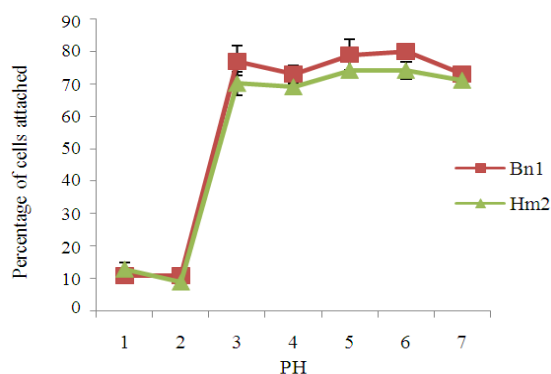


Fig. 4. Effect of pH on adhesion of Bn1 and HM2 to resistant rice starch. Error bars indicate ± 1 standard deviation from the mean (n=3)

3.5. Adhesion Mechanisms

Assessment the contributions of a group of possible adhesion mechanisms to adhesion of probiotic bacteria to resistant rice starch were done as mention at (Table 1). Several growth media were used to test their effects on adhesion. Adhesion factors in fresh or spent extracellular medium did not play a role in adhesion, since washing the cells and resuspending them in buffer, fresh medium, or pepsin-treated spent medium did not inhibit adhesion (Fig. 2).

The effect of hydrophobic interactions was studied by addition of Tween 80 which, did not inhibit adhesion. As did adding 0.5 M NaCl to similarly reduce electrostatic interactions between the starch and the bacteria also failed to inhibit adhesion (Fig. 2).

The role of bacterial proteins in adhesion was studied by using proteolytic and chemical treatments aimed at reducing adhesion. Pretreatment of Bn1 and Hm2 bacteria with pepsin at pH 2.0 completely inhibited adhesion to the starch. To determine if the reduction in adhesion observed after treatment of the cells with pepsin at pH 2.0 was due to protease activity or simply to low pH, the cells were also treated with proteinase K at pH 7.0. This treatment also resulted in a significant reduction in the level of adhesion Bn1 and Hm2 to RRS to 13 and 9% Respectively (Fig. 2). However, treatment of the starch with the protease (pepsin) did not significantly reduce adhesion (Fig. 2), suggesting that the cells were not adhering to any proteins or peptides associated with the starch granules.

The effect of monosaccharides, disaccharides and polysaccharides on bacterial adhesion to rice starch is variable, with the degree of inhibition increasing with molecular size. Adhesion was not inhibited clearly by glucose, sucrose, lactose, or maltose. However, both amylose and amylopectin inhibited adhesion to approximately the same extent (Fig. 3).

3.6. Adhesion under Simulated Upper Digestive Conditions

The influence of digestive tract conditions on bacterial adhesion to rice starch was examined. At pH more than 4.0 (Fig. 4) and presence of bile (Fig. 5) have not affected on adhesion, while pH from 2.0 to 3.0 and protease showed a clear effect on adhesion, so the adhesion will not be preserved during the passage through the stomach. Moreover, treatment of the starch granules with pancreatin led to reduced adhesion capacity (Fig. 5).

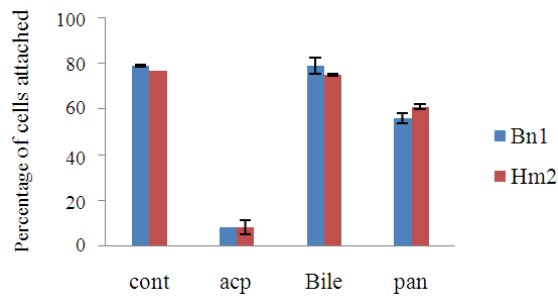


Fig. 5. Influence of upper gastrointestinal conditions (acp acid plus pepsin, bile, pan pancreatic) on adhesion of Bn1 and HM2 to resistant rice starch Error bars indicate ± 1 standard deviation from the mean ($n = 3$)

4. DISCUSSION

Using probiotics as health supplements became widespread, since substitution the use of chemical supplements or antibiotic growth promoters in animals by probiotics (Kosin and Rakshit, 2006). Oral administration of *Lactobacillus* and Bifidobacterium is able to adjusting microbial balance in the intestine (Shah, 2006). Loosing probiotic during processing is the most important technological obstacle that must to be solved. Produce large enough amounts of viable and stable probiotic cultures has been technological challenge for industry. Some studies demonstrated that high levels of viable probiotics are recommended in probiotic foods to be functional (Galdeano and Perdigon, 2004).

Prebiotics like resistant starch can be arrived to the colon with out digestion during passage through the human small intestine (Cummings and Macfarlane, 1997). Resistant starch has a suitable surface for adherence of the probiotics to the starch granule during processing, storage and transit through the upper regions of the gastrointestinal tract (Crittenden *et al.*, 2001).

It was reported that adhesion of different species of probiotics such as *Bifidobacterium* spp to native maize, potato, oat and barley starch granule showed that starch adhesion was not characteristic of all the *Bifidobacteria* tested (Crittenden *et al.*, 2001). Our finding indicated that probiotic bacteria isolated from the various source showed different adhesion level to resistant starch extracted from three type of rice using a co-sedimentation assay. Ten species of bacteria adhered to rice starch granules within 60 min of exposure to the granules. Bn1 and HM2 adhered well to rice starch with a total of 79 and 77% of the cells adhering (highly adherent). YN = 70, CY = 48, FY = 55, HM1 = 61, 5, HM3 = 65 and HM4 = 50, 5% species adhered less well (moderate adherent). YD = 20% and Bn2 = 18% species

adhered less than 40% (Poorly adherent), adhesion to starch granules was measured by a co sedimentation assay.

There is no any effect by extracellular proteins in the adhesion, this fact appeared clearly when treating the spent growth medium with pepsin did not change in values of adhesion of probiotic to rice resistant starch (**Fig. 2**). Extracellular components have been played an important role in the adhesion of *Lactobacillus fermentum* and *L. acidophilus* to human intestinal cells (Conway and Kjelleberg, 1989; Coconnier *et al.*, 1992).

In this study did not notice any effective difference in values of adhesion of probiotics which already have treated with Tween 80 to rice resistant starch (**Fig. 2**), that agreed with previous work by Fernando *et al.* (2011) which; studied attachment of *Bifidobacteria* and *Lactobacilli* to rice fiber fractions. Hwang *et al.* (2008) have Indicated in their study that adding Tween 80 can increase portability of bacterial adhesion to fiber in animal feed. While the results showed decrease portability of bacterial adhesion to fiber in animal feed in the previous study by (Lee *et al.*, 2007). Tween 80 is one of the important food additives (Hwang *et al.*, 2008).

Addition of 0.5 M NaCl did not affect adhesion of probiotic to rice resistant starch (**Fig. 2**), that agreed with previous work by Crittenden *et al.* (2001). while another study performed by (Niderman-Meyer *et al.*, 2009) showed, reduction in adhesion of vibrio cholerae to granular starch by adding 0.5 M NaCl.

Primarily responsible for adhesion of probiotic bacteria to starch granules is cell surface proteins or glycoproteins belong to bacteria not to starch. where the treatment of the resistant starch with the protease did not cause a significant reduction in adhesion ($p < 0.05$) (**Fig. 2**) however, treatment of bacteria with pepsin and pancreases caused a significant reduction. This is agreed with two studies which have conducted by (Bernet *et al.*, 1993; Imam and Harry-O'Kuru, 1991; Chauviere *et al.*, 1992). There is a different effect of saccharides on adhesion of probiotic to rice resistant starch (**Fig. 3**). Polysaccharides inhibited adhesion compared with monosaccharides and disaccharides (**Fig. 3**). This indicates that adhesive proteins have a stronger affinity for larger molecular size of starch polymer chains. Our finding have had agreed strongly with results of Imam and Harry-O'Kuru (1991) who studied the adhesion of *Lactobacillus amylovorus* with corn starch.

The influencing of the digestive conditions on adhesion of probiotic bacterial to rice starch was studied by a set of tests. Medium pH was first factor that has been studied and results showed that neither probiotic bacteria nor resistant rice starch granules are affected by pH (**Fig. 4**). This accorded with previous studies which were showed that a $pH > 4.0$ do not affect on the adhesion

of *Bifidobacteria* to Hylon VII starch (Crittenden *et al.*, 2001) or on adherence of *V. alginolyticus* to chitin (Pruzzo *et al.*, 1996). So pH gradient in the Upper digestive tract does not constitute an obstacle to bacterial adhesion with starch granules.

One beneficial effect that has been resulted from probiotics is a reduction in serum cholesterol levels, due to an enzymatic deconjugation of bile acids (Topping, 1991; Corzo and Gilliland, 1999). On the other hand, deconjugated free bile salt inhibits probiotics. Previous studies have noted that presence of resistant starch promoted survival of *Bifidobacteria* in the intestinal tract of rats even though, in the presence of bile and low pH (Wang *et al.*, 1999). Although present study showed that bile did not influence bacterial adhesion to rice starch. While, treatment of the starch granules with pancreatin has reduced the ability of interdependence between the starch and bacteria. Also the study have done by (Niderman-Meyer *et al.*, 2009) found that treatment of the starch granules with pancreatin has reduced the ability of binding of Hylon VII starch granules and *Bifidobacterium* strains.

Adhesion to resistant starch can be considered as a better methods to increase Probability of survival of probiotic bacteria through the digestive tract.

5. CONCLUSION

The present study focused on ability of LAB isolated from different sources with potential probiotic properties to adhere to several resistant rice starch granules, to achieve a preliminary comprehension of the adhesion mechanisms. The adhesion level was measured by a co-sedimentation assay. In addition, this study had been shown that probiotic isolates have different level of attachment to several type of resistant rice starch. The adhesion of probiotic bacteria to starch appeared as a result of presence of a specific cell surface protein(s) instead of nonspecific hydrophobic or electrostatic interactions. In an vitro gastric model, adhesion to starch may not be influenced by bile but, adhesion was inhibited both by the action of protease and at pH values of <3 (the binding capacity was reduced by low pH and acid pepsin solutions).

One of the solutions to ensure adhesion of probiotic bacteria to resistant starch during passage through the gastrointestinal tract is microencapsulation, so it may be possible to exploit adhesion of probiotic bacteria to starch granules in microencapsulation technology and for synbiotic food applications such as bakery products.

6. ACKNOWLEDGEMENT

We would like to thank Faculty of Science and Technology, Universiti Sains Islam Malaysia for their support.

7. REFERENCES

- Anal, A.K. and H. Singh, 2007. Recent advances in microencapsulation of probiotics for industrial applications and targeted delivery. Trends Food Sci. Technol., 18: 240-251. DOI: 10.1016/j.tifs.2007.01.004
- Bernet, M.F., D. Brassart, J.R. Neeser and A.L. Servin, 1993. Adhesion of human *Bifidobacterial* strains to cultured human intestinal epithelial cells and inhibition of enteropathogen-cell interactions. Applied Environ. Microbiol., 59: 4121-4128.
- Chauviere, G., M.H. Coconnier, S. Kerneis, J. Fourniat and A.L. Servin, 1992. Adhesion of human *Lactobacillus acidophilus* strain LB to human enterocyte-like Caco-2 cells. Microbiology, 138: 1689-1696. DOI: 10.1099/00221287-138-8-1689
- Coconnier, M.H., T.R. Klaenhammer, S. Kerneis, M.F. Bernet and A.L. Servin, 1992. Protein-mediated adhesion of *Lactobacillus acidophilus* BG2FO4 on human enterocyte and mucus-secreting cell lines in culture. Applied Environ. Microbiol., 58: 2034-2039. PMID: 1622282
- Conway, P.L. and S. Kjelleberg, 1989. Protein-mediated adhesion of *Lactobacillus fermentum* strain 737 to mouse stomach squamous epithelium. J. Gen. Microbiol., 135: 1175-1186. PMID: 2559944
- Corzo, G. and S.E. Gilliland, 1999. Bile salt hydrolase activity of three strains of *Lactobacillus acidophilus*. J. Dairy Sci., 82: 472-80. PMID: 10194664
- Crittenden, R., A. Laitila, P. Forssell, J. Matto and M. Saarela *et al.*, 2001. Adhesion of *Bifidobacteria* to granular starch and its implications in probiotic technologies. J. Microbiol. Technol., 67: 3469-3475. PMID: 11472921
- Cummings, J.H. and G.T. Macfarlane, 1997. Colonic microflora: Nutrition and health. Nutrition, 13: 476-478. PMID: 9225346
- Fernando, W., S. Flint, S. Charles, K.D.S. Ranaweera and A. Bamunuarachchi, 2011. Environmental factors affecting the adhesion of probiotics to rice fiber fractions. Food Dig., 2: 26-36. DOI: 10.1007/s13228-011-0012-7

- Galdeano, C.M. and G. Perdigon, 2004. Role of viability of probiotic strains in their persistence in the gut and in mucosal immune stimulation. *J. Applied Microbiol.*, 97: 673-681. PMID: 15357716
- Hwang, H.I.I., C.H. Lee, S.W. Kim, H.G. Sung and S.S. Lee *et al.*, 2008. Effects of mixtures of Tween 80 and cellulolytic enzymes on nutrient digestion and cellulolytic bacterial adhesion. *Asian-Aus. J. Anim. Sci.*, 21: 1604-1609.
- Imam, S.H. and R.E. Harry-O’Kuru, 1991. Adhesion of *lactobacillus amylovorus* to insoluble and derivatized cornstarch granules. *Applied Environ. Microbiol.*, 57: 1128-1133. PMID: 16348460
- Kheadr, E.E., 2006. Impact of acid and oxgall on antibiotic susceptibility of probiotic *Lactobacilli*. *Afr. J. Agric. Res.*, 1: 172-181.
- Kosin, B. and S.K. Rakshit, 2006. Microbial and processing criteria for production of probiotics: A review. *Food Technol. Biotechnol.*, 44: 371-379.
- Lee, C.H., H.G. Sung, M. Eslami, S.Y. Lee and J.Y. Song *et al.*, 2007. Effects of Tween 80 pretreatment on dry matter disappearance of rice straw and cellulolytic bacterial adhesion. *Asian Aus. J. Anim. Sci.*, 20: 1397-1401.
- Mortazavian, A.M., A. Azizi, M.R. Ehsani, S.H. Razavi and S.M. Mousavi *et al.*, 2008. Survival of encapsulated probiotic bacteria in Iranian yogurt drink (Doogh) after the product exposure to simulated gastrointestinal conditions. *Milchwissenschaft*, 63: 427-429.
- Niderman-Meyer, O., T. Zeidman, E. Shimoni and Y. Kashi, 2009. Mechanisms involved in governing adherence of *vibrio cholerae* to granular starch. *Applied Environ. Microbiol.*, 76: 1034-1043. DOI: 10.1128/AEM.01533-09
- Pongjanta, J., A. Utaipatanacheep, O. Naivikul and K. Piyachomkwan, 2008. Enzymes-Resistant Starch (RS III) from pullulanase- debranched high amylose rice starch. *Kasetsart J. Nat. Sci.*, 42: 198-205.
- Pruzzo, C., A. Crippa, S. Bertone, L. Pane and A. Carli, 1996. Attachment of *Vibrio alginolyticus* to chitin mediated by chitin-binding proteins. *Microbiology*, 142: 2181-2186. PMID: 8760933
- Sajilata, M.G., R.S. Singhal and P.R. Kulkarni, 2006. Resistant starch-a review. *Comprehensive Rev. Food Sci. Food Safety*, 5: 1-17. DOI: 10.1111/j.1541-4337.2006.tb00076.x
- Shah, N.P., 2006. Microorganisms and Health Attributes (Probiotics). In: *Manufacturing Yogurt and Fermented Milks*, Chandan, R.C. (Ed.), Blackwell Publishing Professional, Iowa, USA., pp: 341-354.
- Topping, D.L., 1991. Soluble fiber polysaccharides: Effects on plasma cholesterol and colonic fermentation. *Nutr. Rev.*, 49: 195-203. PMID: 1658695
- Wang, L. and Y.J. Wang, 2001. Comparison of protease digestion at neutral pH with alkaline steeping method for rice starch isolation. *Cereal Chem.*, 78: 690-692. DOI: 10.1094/CCHEM.2001.78.6.690
- Wang, X., I.L. Brown, A.J. Evans and P.L. Conway, 1999. The protective effects of high amylose maize (amylomaize) starch granules on the survival of *Bifidobacterium* spp. in the mouse intestinal tract. *J. Applied Microbiol.*, 87: 631-639. PMID: 10594702
- Yavuzdurmaz, H., 2007. Isolation, characterization, determination of probiotic properties of lactic acid bacteria from human milk. Thesis. Izmir Institute of Technology.