(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
9 June 2011 (09.06.2011)

PCT

(10) International Publication Number
WO 2011/068398 A1

(51) International Patent Classification:
A61K 35/74 (2006.01) *C12N 1/20* (2006.01)
A61P 1/00 (2006.01)

(21) International Application Number:
PCT/MY2010/000306

(22) International Filing Date:
29 November 2010 (29.11.2010)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
PI20097032 1 December 2009 (01.12.2009) MY

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

— *with sequence listing part of description (Rule 5.2(a))*

(54) **Title**: PROBIOTIC COMPOSITION OBTAINED FROM LACTOBACILLUS PLANTARUM STRAINS UL4, TL1, RS5, R111, RG14 OR COMBINATIONS THEREOF

(57) **Abstract**: Probiotic compositions obtained from Lactobacillus Plantarum strains UL4, TL1, RS5, RI 1 1, RG11, RG14 or combinations thereof comprising metabolites including bacteriocin, vitamin and organic acid! Administering the probiotic compositions to animals to: 1) improve their growth performance, 2) reduce serum cholesterol levels, 3) change intestinal microflora (by reducing infection by enterococci, Escherichia coli), and 4) increase intestinal villi height, crypt depth and volatile fatty acids.

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PROBIOTIC COMPOSITION FOR NUTRACEUTICAL PRODUCT

FIELD OF INVENTION

The present invention relates a naturally occurring source of metabolites produced by probiotic bacterial. Moreover, the present invention discloses the use of metabolites derived from *Lactobacillus* spp., in nutraceutical products.

BACKGROUND OF INVENTION

Lactobacillus is a genus of Lactic acid bacteria (LAB) found in the intestinal tracts of mammals, green plants, milk and fermenting foods. Lactic acid is produced by these bacteria via carbohydrate fermentation; thus, lactobacilli have been introduced into the manufacturing of food products, such as yogurt and cheese, to enhance their quality and stability. In addition, lactobacilli form an important part of the internal microbial flora in humans and other animals. The natural adaptation of many (LAB to the gut environment and the antimicrobial substances such as organic acids and bacteriocins produced has provided a competitive advantage over other microorganisms (Guerra *et. al.,* 2007; Bogovič Matijašić *et al.*, 2004; Salminen *et al.*, 1998). The administration of a probiotic bacteria and their metabolites has found to be an effective way to promote body weight and feed conversion in farm animals (Denli *et al.*, 2003; Loh *et al.*, 2003; Abe *et al.*, 1995). Furthermore in comparison to live probiotics, metabolites with proteinacious nature are biodegradable, environmental friendly, ease in application, storage and transportation. Bacteriocins are biologically active protein moieties displaying an antimicrobial action against Gram-positive species and possibly food spoilers and pathogens. The inhibitory spectrum of bacteriocins is restricted to Gram-positive bacteria, but several bacteriocins produced by LAB are active against food spoilage and food-borne pathogenic microorganisms, including *Bacillus cereus, Clostridium botulinum, Listeria monocytogenes, Staphylococcus aureus* etc (De Vuyst and Vandamme, 1994). Bacteriocins can also be used in food industry as biopreservative. Nisin, a known bacteriocin produced by *Lactococcus lactis* subsp. *lactis,* is commonly used as biopreservative in dairy products to inhibit the growth of foodborne pathogens (Hill,

1995). Besides being the end-products of carbohydrate fermentation, lactic and acetic acids also play an important role as inhibitory agents (De Vuyst and Vandamme, 1994). The accumulation of acidic end-products and the concomitant low pH results in a wide inhibitory spectrum including both Gram-positive and Gram-negative bacteria (Blom and M□rtvedt, 1991).

The present invention relates to nutraceutical product containing *Lactobacillus* sp. metabolites and a method of using this supplement. More specifically, the nutraceutical product of the present invention includes bacteriocins and organic acids, such as acetic acid and lactic acid. The present invention also has the ability to inhibit various food-borne pathogens. Health promoting effect of reduction in serum cholesterol level is another aspect of the present invention.

Unexpectedly, the inventors have noted that the invention of at least six strains of specific probiotic microorganisms is found to be most effective, particularly in adults, for monitoring of serum cholesterol level; and monitoring animal performance such as in the management of growth, in the treatment of diseases caused by intestinal microflora, providing the capability to increase villi height, crypt depth and volatile fatty acids of an intestine.

The present invention yet further relates to the use of at least one strain of a microorganism, preferably a lactic acid bacterium, preferably a probiotic bacterium, such as *Lactobacillus* sp. (for example *Lactobacillus* spp. of UL4, TL1, RS5, RI11, RG11, RG14 strains), in monitoring of serum cholesterol level; and monitoring animal performance such as in the management of growth, in the treatment of diseases caused by intestinal microflora, providing the capability to increase villi height, crypt depth and volatile fatty acids of an intestine.

SUMMARY OF INVENTION

Accordingly, the present invention relates a probiotic composition obtained from *Lactobacillus* sp of UL4, TL1, RS5, RI11, RG11, RG14 or a combination thereof are deposited at the BIOTEC Culture Collection (BCC), BIOTEC Central Research Unit of Thailand. Moreover, the composition includes metabolites such as bacteriocin, vitamin (Vitamin B), organic acids (such as formic, acetic and lactic acid). The *Lactobacillus* sp. strains UL4, TL1, RS5, RI11, RG11, RG14 having an effective amount of bacteriocin between 50 AU/ml and 800AU/mL. Also, the *Lactobacillus* sp. UL4, TL1, RS5, RI11, RG11, RG14 in combination having an effective amount of bacteriocin between 50 AU/ml and 800AU/mL. It is said that the metabolite(s) provides nutritionally and/or pharmaceutically acceptable carrier. Indeed, the probiotic composition provides an effective amount of probiotic in a nutraceutical product and the probiotic composition provides a use as a medicament.

In addition, the probiotic composition in the present invention prevents or reduces an infection with Vancomycin resistant enterococci (VRE), *Listeria monocytogenes, Bacillus cereus, Streptococcus pneumonia, Staphylococcus aureus, Salmonella typhimurium* and *Escherichia coli* of an animal, including a human.

Yet, another aspect of the present invention relates to the use of probiotic composition for the manufacturing of a medicament for the probiotic treatment of an animal, including a human. Furthermore, the invention discloses an effective amount of the probiotic composition in the preparation of a medicament for reducing/monitoring serum cholesterol level in an animal including human. Also, a preferred embodiment of the present invention relates to an effective amount of the probiotic composition in the preparation of a medicament for increasing animal performance such as in the management of growth of an animal, including a human.

In addition, the present invention discloses an effective amount of the probiotic composition in the preparation of a medicament in the treatment of diseases caused by intestinal microflora. Lastly, this invention describes an effective amount of the probiotic composition in the preparation of a medicament in the providing the capability to improve gut health by increasing villi height, crypt depth and volatile fatty acids in an intestine.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will be fully understood from the detailed description given herein below and the accompanying drawings which are given by way of illustration only, and thus are not limitative of the present invention, wherein:

Figure 1 is a picture of antagonistic activity of metabolites against various Gram-positive and Gram-negative bacteria determined by agar well diffusion assay.

Figure 2 is representing the effect of direct fed of metabolites on population of *Enterobacteriaceae* bacteria in faecal samples.

Figure 3 is representing the effect of direct fed of metabolites on population of lactic acid bacteria in different intestinal regions.

Figure 4 is representing the effect of direct fed of metabolites on population of *Enterobacteriaceae* bacteria in different intestinal regions.

Figure 5 is representing the effect of direct fed of metabolites on population of lactic acid bacteria and *Enterobacteriaceae* bacteria in intestinal digesta.

Figure 6 is representing the effect of direct fed of metabolites on crypt depth of different small and large intestinal regions.

Figure 7 is representing the effect of direct fed of metabolites on concentration of short chain fatty acids in Week-4 faecal samples.

Figure 8 is a graph representing the Effect of direct fed of metabolites on serum cholesterol level of rats after feeding trial.

DETAILED DESCIPTION OF THE INVENTION

An object of the invention is the use of probiotic bacteria for the manufacturing of a food product, feed product, nutraceutical product(s) (such as dietary supplement), natural remedy, pharmaceutical active formulation and medicinal product to be used for controlling weight gain, preventing obesity, , reducing fat deposition, improving energy metabolism, and treating obesity.. The term "nutraceutical" as used herein denotes usefulness in both the nutritional and pharmaceutical field of application. Thus, the novel probiotic composition can find use as supplement to food and beverages, and as pharmaceutical formulations for enteral or parenteral application which may be solid formulations such as capsules or tablets, or liquid formulations, such as solutions or suspensions.

Accordingly, the present invention provides novel metabolites possessing probiotic characteristics for use as nutraceutical product(s) (such as dietary supplement). The nutraceutical products includean effective amount of a naturally occurring source of *Lactobacillus* sp. metabolite is provided. Preferably, the nutraceutical products include at least metabolites with 800 AU/mL of inhibitory activity. The subject invention provides methods for improving host health. In specific embodiments, the invention provides methods for accelerating and/or augmenting host growth; reduce serum cholesterol concentration; and overall health in host.

This object is achieved in that the probiotic bacteria being selected from the group consisting of LAB especially *Lactobacillus* sp. of UL4, TL1, RS5, RI11, RG11, RG14 and the combination of 6 metabolites deposited at the BIOTEC Culture Collection (BCC), BIOTEC Central Research Unit of Thailand.

Also, the present invention is an isolated and purified bacteriocin characterized in that it shows reduce the Enterobacteriaceae population in intestine and had significantly increased the crypt depth of small intestine and caecum, which might play an essential role in replenish the integrity of villous to maintain absorptive and digestive function. Said bacteriocin can be obtainable from *Lactobacillus* sp. of UL4, TL1, RS5, RI11, RG11,

RG14 and the combination of 6 metabolites deposited at the BIOTEC Culture Collection (BCC), BIOTEC Central Research Unit of Thailand.

Said bacteriocin is preferably obtainable from strains of UL4, TL1, RS5, RI11, RG11, RG14 by the following steps: Obtaining a cell-free supernatant from a fermentation culture comprising *Lactobacillus plantarum* I-UL4, TL1, RS5, RI11, RG11, RG14, 'A purification step to obtain an at least partially purified bacteriocin. Said purification steps preferably comprise of precipitation, ultrafiltration and column chromatography. The purification step can further comprise an salt or organic solvent extraction/precipitation step, followed by ultrafiltration technique using < 10 kDa , membrane to fractionate the bioactive molecule comprising bacteriocin to be further purified by column chromatography consisting of ion-exchange and gel filtration chromatography. The bacteriocin activity of each purification step was determined by agar well diffusion assay and the bacteriocin activity was expressed as AU/ml.

Another aspect of the present invention is a bacteriocin or a probiotic composition of the present invention for use as a probiotic.

Another aspect of the present invention is a bacteriocin or a probiotic composition of the present invention for use as a medicament. Wherein the medicament is use in the monitoring and treatment of serum cholesterol level in a person in need of such treatment. Yet, the medicament is also use in the providing means of animal performance such as in management of growth, in the treatment of diseases caused by intestinal microflora, providing the capability to improve the intestinal health by increasing the villi height, crypt depth and volatile fatty acids of small intestine.

Another aspect of the present invention is a bacteriocin or a probiotic composition of the present invention for the prevention of faecal and intestinal microbial population, namely LAB and *Enterobacteriaceae* (ETB). Another aspect of the present invention is a bacteriocin or a probiotic composition of the present invention for curing an infection with Vancomycin resistant enterococci (VRE), *Listeria monocytogenes, Bacillus cereus, Streptococcus pneumonia, Staphylococcus aureus, Salmonella typhimurium* and *Escherichia coli.* **Table 1** represents inhibitory zone score ranking of 63 combinations of

metabolites produced by the 6 strains (*Lactobacillus plantarum* I-UL4, TL1, RS5, RI11, RG11, RG14).

Table 1. Inhibitory zone score ranking of 63 combinations of metabolites produced by 6 strains of *Lactobacillus plantarum* against *Escherichia coli, Listeria monocytogenes, Salmonella typhimurium,* Vancomycin resistant enterococci (VRE) and *Pediococcus acidilactici*

Trmt	Com[1]	*E. coli*		*L. monocytogenes*		*S. Typhimurium*		VRE		*P. acidilactici*		Score[4]
		In. zone[2]	Rank[3]	In. zone	Rank	In. zone	Rank	In. zone	Rank	In. zone	Rank	
56	3456	14.33 ± 1.20^{bdac}	5	15.33 ± 1.45^{ba}	2	$14.33 \pm 0.88^{ebdhagcf}$	9	$10.67 + 0.33^{ba}$	2	14.67 ± 0.33^{bdc}	5	292
37	256	12.67 ± 0.88^{ebdacf}	10	15.33 ± 0.33^{ba}	2	16.67 ± 0.67^{bac}	3	$7.67 + 0.33^{igh}$	9	16.00 ± 0.00^{a}	1	290
36	246	15.33 ± 1.86^{bac}	3	14.00 ± 0.56^{ebdacf}	6	15.67 ± 0.67^{ebdac}	5	$7.67 + 0.33^{igh}$	9	15.33 ± 0.33^{bac}	3	289
54	2356	13.67 ± 1.45^{ebdac}	7	15.00 ± 0.58^{bac}	3	$14.33 \pm 0.67^{ebdhagcf}$	9	$11.33 + 0.33^{a}$	1	14.33 ± 0.33^{dc}	6	289
57	12345	14.00 ± 1.00^{ebdac}	6	14.33 ± 0.33^{ebdac}	5	$13.67 \pm 0.33^{ebdhagcf}$	11	$9.67 + 0.33^{bcd}$	3	15.33 ± 0.33^{bac}	3	287
63	123456	13.67 ± 0.67^{ebdac}	7	14.67 ± 0.67^{ebdacf}	4	$13.67 \pm 0.33^{ebdhagcf}$	11	$9.67 + 0.33^{bcd}$	3	15.33 ± 0.67^{bac}	3	287
32	234	13.33 ± 1.45^{ebdac}	8	14.67 ± 2.60^{bdac}	4	$14.00 \pm 0.58^{ebdhagcf}$	10	$8.33 + 0.33^{efgh}$	7	16.00 ± 0.00^{a}	1	285
49	1346	15.67 ± 0.88^{ba}	2	$12.67 \pm 0.33^{ebdhagcf}$	10	15.33 ± 1.86^{ebdagcf}	6	$8.00 + 0.58^{ifgh}$	8	15.00 ± 0.00^{bdac}	4	285
55	2456	12.67 ± 1.33^{ebdacf}	10	14.00 ± 0.58^{ebdacf}	6	15.00 ± 0.58^{ebdagcf}	7	$10.67 + 0.33^{ba}$	2	14.67 ± 0.33^{bdc}	5	285
25	126	12.33 ± 1.76^{ebdcf}	11	14.00 ± 3.06^{ebdacf}	6	16.33 ± 1.45^{bdac}	4	$8.67 + 0.33^{efgd}$	6	15.00 ± 0.00^{bdac}	4	284
44	1236	15.33 ± 1.45^{bac}	3	$12.67 \pm 0.33^{ebdhagcf}$	10	$14.33 \pm 1.86^{ebdhagcf}$	9	$8.33 + 0.33^{efgh}$	7	15.67 ± 0.33^{ba}	2	284
53	2346	13.33 ± 1.33^{ebdac}	8	16.00 ± 0.58^{a}	1	$14.33 \pm 0.88^{ebdhagcf}$	9	$8.33 + 0.33^{efgh}$	7	14.33 ± 0.33^{dc}	6	284
62	23456	14.00 ± 0.58^{ebdac}	6	14.33 ± 0.33^{ebdac}	5	12.67 ± 0.33^{edhgcf}	14	$9.67 + 0.33^{bcd}$	3	15.33 ± 0.67^{bac}	3	284
52	2345	14.33 ± 0.88^{bdac}	5	14.67 ± 0.88^{bdac}	4	$13.67 \pm 0.67^{ebdhagcf}$	11	$8.00 + 0.00^{ifgh}$	8	15.00 ± 0.00^{bdac}	4	283
5	5	12.67 ± 1.20^{ebdac}	10	14.67 ± 0.33^{bdac}	4	$14.67 \pm 2.67^{ebdhagcf}$	8	$7.33 + 0.33^{ih}$	10	16.00 ± 0.00^{a}	1	282
42	1234	13.67 ± 0.33^{ebdac}	7	11.00 ± 1.53^{edhgf}	14	17.67 ± 1.20^{ba}	2	$8.00 + 0.00^{ifgh}$	8	15.67 ± 0.33^{ba}	2	282
51	1456	14.00 ± 1.53^{ebdac}	6	14.67 ± 0.33^{bdac}	4	$14.33 \pm 0.88^{ebdhagcf}$	9	$8.00 + 0.00^{ifgh}$	8	14.33 ± 0.33^{dc}	6	282

[1]63 combinations from 6 strains of *L. plantarum* UL4, TL1, RS5, RI11, RG14 and RG11, which were numbered 1, 2, 3, 4, 5 and 6 respectively.
[2]Means (mean of inhibitory zone diameter ± SEM) in the same column with common superscripts are non-significantly different
[3]Ranking of inhibitory zone based on inhibitory zone diameter against single indicator strain.
[4]Score is the sum of single indicator score as a subtraction of 63 and ranking number (score = 63 – rank). The combination with higher score has stronger inhibitory activity against 4 above-mentioned indicator strains. It was descendible arranged in the column

Another aspect of the present invention is the use of the bacteriocin or a probiotic composition according to the present invention for the manufacturing of a medicament for the probiotic treatment of an animal, including a human.

Another aspect of the present invention is the use of the bacteriocin or a probiotic composition according to the present invention for the preparation of a medicament for the prevention or inhibition of an infection with faecal and intestinal microbial population, namely LAB and *Enterobacteriaceae* (ETB) in an animal, including a human.

It is therefore an object of the present invention to provide compositions for use in supplementing the mammalian diet comprising dried bacteria, such as *Lactobacillus* sp. of UL4, TL1, RS5, RI11, RG11, RG14 and the combination of 6 metabolites that are stable upon storage at room temperature.

It is another object of the present invention to provide compositions comprising living cell of *Lactobacillus* sp. of UL4, TL1, RS5, RI11, RG11, RG14 and the combination of 6 metabolites cultures that are useful in the preparation of commercially viable food or food supplement products.

It is a further object of the present invention to provide compositions for use as a nutraceutical products comprising *Lactobacillus* sp. of UL4, TL1, RS5, RI11, RG11, RG14 and the combination of 6 metabolites, that are useful for the manufacturing and processing of foods, and dietary food supplementation products. It is another object of the present invention to provide composition comprising living bacterial cultures, such as *Lactobacillus* sp. of UL4, TL1, RS5, RI11, RG11, RG14 and the combination of 6 metabolites that are useful in food preservation.

It is a further object of the present invention to provide compositions for use as a nutraceutical products comprising *Lactobacillus* sp. of UL4, TL1, RS5, RI11, RG11, RG14 and the combination of 6 metabolites cultures to the human diet that is useful in promoting the health and well being of humans having the condition of lactose intolerance.

BEST MODE TO CARRY OUT THE INVENTION

Before the present invention is further described, it is to be understood that this invention is not limited to particular embodiments described, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims. When a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limit of that range and any other stated or intervening value in that stated range, is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges, and are also encompassed within the invention, subject to any specifically excluded limit in the stated range. When the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

EXAMPLES

The inventors in the present invention demonstrates methods to evaluate the effectiveness of metabolites produced by *Lactobacillus* sp. by antagonistic activity, on growth performance, intestinal microflora and histomorphological changes, alteration of short chain fatty acids and serum cholesterol level in animal rat. The metabolites were prepared by inoculated and incubated the bacteria culture into MRS broth anaerobically. A total of 64 rats were used in this study. The rats were fed with Control (without metabolites), and metabolites produced by respective *Lactobacillus* sp. of UL4, TL1, RS5, RI11, RG11, RG14 and the combination of 6 metabolites (at 1:1 ratio) at the final dose of 800 AU/mL daily. All rats were healthy and survived throughout the 28 d of feeding trial. No adverse effects were shown on the health status, growth or development of the animals. Results obtained further convinced the safe consumption of metabolite.

In the measurement of faecal microbial population, high and constant population of LAB was detected in the faecal samples of all treatment groups with no significance difference ($P > 0.05$) was observed. In contrast, low and fluctuated population of ETB was detected.

The results obtained in this study suggested that the intestinal LAB are susceptible to the presence of metabolites, but affected the ETB population.

The lowest ETB population was detected in intestinal digesta of Control, RS5 and RI11 groups. Results obtained might have further confirmed the ability of metabolite to enhance population of LAB, while reducing the population of ETB. As in large intestinal regions, all treatment groups demonstrated higher LAB population in caecum, when compared with Control group.

It is said that the metabolites derived from *Lactobacillus* sp. of UL4, TL1, RS5, RI11, RG11, RG14 had significantly increased the crypt depth of small intestine and caecum. Combination of six metabolites (Combination group) had successfully increased the villous height and crypt depth of ileum. While in caecum of large intestine, all treatment groups demonstrated larger crypt depth than Control group (except for TL1 and RG11 groups).

In the assessment of volatile fatty acids (VFA) detected in faeces, no significant difference ($P>0.05$) was observed in the production of VFA, except for butyric acid. Results suggesting that supplementation of metabolites did not alter the metabolic activity in the gastrointestinal tract.

Rats fed with metabolites showed more than 4.5 % of reduction in serum cholesterol level. Metabolites RS5, RI11 and RG11 had significantly ($P<0.05$) reduced the serum cholesterol by 21.91 %, 15.62 % and 15.23 % respectively. In the *in vitro* antimicrobial assay, these metabolites produced by *Lactobacillus* sp. had exerted strong bacteriocidal inhibition on Gram-positive bacteria, such as Vancomycin resistant enterococci (VRE), *Listeria monocytogenes, Bacillus cereus, Streptococcus pneumonia, Staphylococcus aureus, Salmonella typhimurium* and *Escherichia coli.*

Table 2 provides the effect of direct fed of metabolites on total feed intake, total live weight gained and feed conversion ratio in rats.

Control	**389.40**	±	21.10^{ab}	**61.33**	±	4.26^{ab}	**6.61**	± 0.65^{ab}
UL4	**446.90**	±	14.10^{a}	**52.48**	±	2.99^{b}	**8.71**	± 0.61^{a}
TL1	**375.70**	±	22.80^{b}	**59.30**	±	4.76^{ab}	**6.45**	± 0.43^{ab}
RS5	**413.10**	±	12.00^{ab}	**66.73**	±	4.31^{a}	**6.40**	± 0.50^{ab}
RI11	**378.10**	±	22.10^{b}	**68.61**	±	3.16^{a}	**5.59**	± 0.38^{b}
RG11	**386.30**	±	15.00^{ab}	**49.11**	±	5.47^{b}	**8.90**	± 1.54^{a}
RG14	**403.10**	±	13.80^{ab}	**61.13**	±	4.45^{ab}	**6.80**	± 0.45^{ab}
Combination	**425.60**	±	30.40^{ab}	**61.85**	±	5.18^{ab}	**7.25**	± 0.86^{ab}

Notes:

- Values are mean ± standard error mean (SEM), n = 8.
- ^{ab} Within a column, values with different superscripts are significantly different at $P<0.05$
- FCR is the total feed intake (g) per total live weight gained (g) of rat in 4 weeks treatment.

Table 3 provides the effect of direct fed of metabolites on serum cholesterol level of rats after feeding trial.

	Week-0			Week-4		
Control	**0.717**	±	$0.039^{a; y}$	**0.685**	±	$0.053^{ab; y}$
UL4	**0.725**	±	$0.038^{a; y}$	**0.625**	±	$0.032^{ab; y}$
TL1	**0.782**	±	$0.018^{a; y}$	**0.744**	±	$0.036^{a; y}$
RS5	**0.707**	±	$0.035^{a; y}$	**0.552**	±	$0.053^{b; z *}$
RI11	**0.750**	±	$0.028^{a; y}$	**0.633**	±	$0.043^{ab; z *}$
RG11	**0.696**	±	$0.024^{a; y}$	**0.590**	±	$0.023^{b; z **}$
RG14	**0.692**	±	$0.030^{a; y}$	**0.643**	±	$0.057^{ab; y}$
Combination	**0.690**	±	$0.024^{a; y}$	**0.632**	±	$0.037^{ab; y}$

Notes:

- Values are mean ± standard error mean (SEM), n = 8.
- [a] Within a colomn, values with different superscripts are significantly different at $P<0.05$.
-Within a row, valucs with astcrisk arc significantly diffcrcnt whcn comparc with Wcck-0, * $p<0.05$; ** $p<0.01$

Table 4 provides the effect of direct fed of metabolites on population of lactic acid bacteria in faecal samples.

	Week-0	Week-1	Week-2	Week-3	Week-4
Control	9.37 ± 0.18 bc; z	10.79 ± 0.27 a; xy	11.27 ± 0.13 a; x	10.16 ± 0.29 bc; y	10.70 ± 0.26 a; xy
UL4	9.00 ± 0.37 c; y	9.30 ± 0.23 c; y	9.39 ± 0.29 cd; y	9.72 ± 0.32 bc; xy	10.47 ± 0.24 ab; xy
TL1	9.45 ± 0.27 bc; y	9.88 ± 0.31 bc; xy	8.32 ± 0.32 ef; z	9.31 ± 0.34 c; y	10.64 ± 0.32 a; x
RS5	9.50 ± 0.34 bc; yz	10.64 ± 0.24 ab; x	9.03 ± 0.30 de; z	10.19 ± 0.23 bc; xy	10.23 ± 0.18 ab; xy
RI11	9.07 ± 0.21 c; y	10.24 ± 0.30 ab; x	8.11 ± 0.32[f] ; z	10.36 ± 0.30 ab; xy	10.26 ± 0.30 ab; x
RG11	9.13 ± 0.33 c; y	10.34 ± 0.20 ab; x	10.14 ± 0.35 bc; x	9.52 ± 0.27 bc; xy	10.27 ± 0.28 ab; x
RG14	9.52 ± 0.27 bc; y	9.86 ± 0.27 bc; y	10.18 ± 0.35 bc; xy	10.18 ± 0.35 bc; xy	10.99 ± 0.14 a; x
Combination	10.69 ± 0.20 a; x	10.61 ± 0.21 ab; x	10.50 ± 0.21 ab; x	11.13 ± 0.29 a; y	10.60 ± 0.29 a; x

Notes:

- Values are mean ± standard error mean (SEM), n = 8.
- [abcdef] Within a column, values with different superscripts are significantly different at $P < 0.05$
- [xyz] Within a row, values with different superscripts are significantly different at $P < 0.05$

CLAIMS

1. A probiotic composition obtained from *Lactobacillus* sp. of UL4, TL1, RS5, RI11, RG11, RG14 or a combination thereof are deposited at the BIOTEC Culture Collection (BCC), BIOTEC Central Research Unit of Thailand, wherein the composition comprises metabolites including:
   bacteriocin,
   vitamin such as Vitamin B, and
   organic acids such as formic, acetic and lactic acid.

2. The probiotic composition according to claim 1, wherein *Lactobacillus* sp. strains UL4, TL1, RS5, RI11, RG11, RG14 having an effective amount of bacteriocin between 50 AU/ml and 800AU/mL.

3. The probiotic composition according to claim 1, wherein obtained from *Lactobacillus* sp. UL4, TL1, RS5, RI11, RG11, RG14 in combination having an effective amount of bacteriocin between 50 AU/ml and 800AU/mL.

4. The probiotic composition comprising metabolites such as in any of the claims 1 to 3 provides nutritionally and/or pharmaceutically acceptable carrier.

5. The probiotic composition as in claim 1 to 4 for use in a nutraceutical product.

6. The probiotic composition as in claim 1 to 4 for use as a medicament.

7. The probiotic composition as in claim 1 or 6 for the prevention or reducing an infection with Vancomycin resistant enterococci (VRE), *Listeria monocytogenes, Bacillus cereus, Streptococcus pneumonia, Staphylococcus aureus, Salmonella typhimurium* and *Escherichia coli* of an animal, including a human.

8. Use of the probiotic composition as in claim 1 or 7 for the manufacture of a medicament for the probiotic treatment of an animal, including a human.

9. Use of an effective amount of the probiotic composition of Claim 1 to 8 in the preparation of a medicament for reducing/monitoring serum cholesterol level in an animal including human.

10. Use of an effective amount of the probiotic composition of Claim 1 to 8 in the preparation of a medicament for increasing animal performance such as in the management of growth of an animal, including a human.

11. Use of an effective amount of the probiotic composition of Claim 1 to 8 in the preparation of a medicament in the treatment of diseases caused by intestinal microflora.

12. Use of an effective amount of the probiotic composition of Claim 1 to 8 in the preparation of a medicament in the providing the capability to improve gut health by increasing villi height, crypt depth and volatile fatty acids in an intestine.

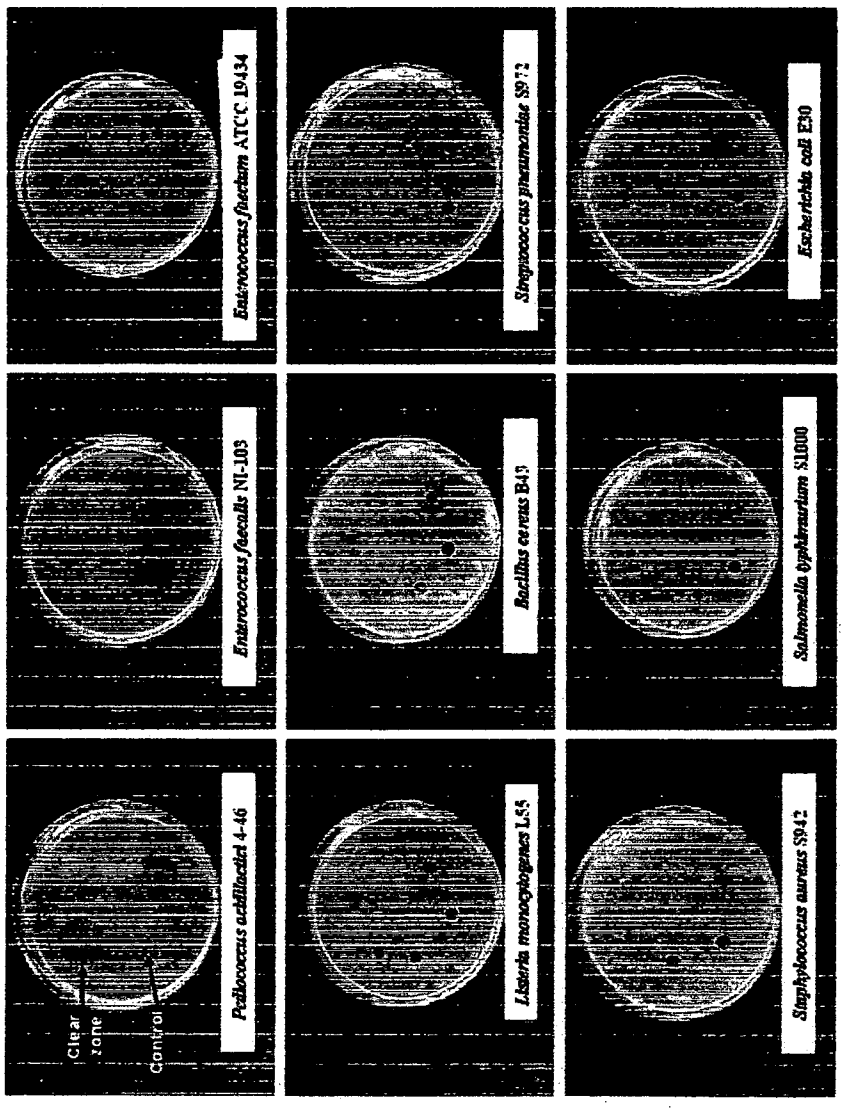


FIGURE 1: Antagonistic activity of metabolites against various Gram-positive and Gram-negative bacteria determined by agar well diffusion assay

FIGURE 2: Effect of direct fed of metabolites on population of *Enterobacteriaceae* bacteria in faecal samples

Metabolites	Population of *Enterobacteriacea* bacteria (Log_{10} CFU/mL) in faecal samples														
	Week-0			Week-1			Week-2			Week-3			Week-4		
Control	5.39	±	$0.20^{ab;\ xy}$	5.61	±	$0.18^{ci\ x}$	4.97	±	$0.15^{ci\ y}$	5.79	±	$0.14^{bi\ x}$	5.66	±	$0.13^{bi\ x}$
UL4	5.38	±	$0.24^{ab;\ y}$	6.20	±	$0.32^{abc;\ x}$	5.38	±	$0.10^{bc;\ y}$	5.88	±	$0.12^{bi\ xy}$	5.69	±	$0.22^{bi\ xy}$
TL1	5.84	±	$0.31^{ai\ x}$	5.91	±	$0.23^{bc;\ x}$	5.35	±	$0.09^{bc;\ xy}$	5.43	±	$0.11^{bc;\ xy}$	4.89	±	$0.18^{ci\ y}$
RS5	4.92	±	$0.13^{bi;\ z}$	5.50	±	$0.26^{ci;\ xy}$	5.25	±	$0.16^{bc;\ yz}$	5.40	±	$0.16^{bc;\ xyz}$	5.81	±	$0.11^{bi\ x}$
RI11	5.36	±	$0.32^{ab;\ xy}$	5.54	±	$0.31^{ci\ xy}$	5.15	±	$0.14^{ci\ y}$	5.31	±	$0.16^{bc;\ xy}$	6.03	±	$0.21^{bi\ x}$
RG11	5.85	±	$0.29^{ai;\ yz}$	6.77	±	$0.38^{ai\ x}$	5.35	±	$0.08^{bc;\ z}$	5.17	±	$0.16^{ci\ z}$	6.32	±	$0.35^{ab;\ xy}$
RG14	5.24	±	$0.21^{ab;\ z}$	6.68	±	$0.30^{ab;\ xy}$	5.88	±	$0.39^{ab;\ yz}$	5.84	±	$0.39^{bi;\ yz}$	6.79	±	$0.20^{ai\ x}$
Combination	5.38	±	$0.20^{ab;\ z}$	6.00	±	$0.18^{abc;\ xyz}$	6.15	±	$0.31^{ai\ xy}$	5.53	±	$0.03^{bc;\ yz}$	6.20	±	$0.27^{ab;\ x}$

Notes:

- Values are mean ± standard error mean (SEM), n = 8.

- ^{abc} Within a column, values with different superscripts are significantly different at $P < 0.05$

- ^{xyz} Within a row, values with different superscripts are significantly different at $P < 0.05$

FIGURE 3: Effect of direct fed of metabolites on population of lactic acid bacteria in different intestinal regions.

Metabolites	Population of lactic acid bacteria (Log_{10} CFU/mL) in different intestinal regions														
	Duodenum			Jejunum			Ileum			Caecum			Colon		
Control	3.80	±	$0.16^{a;\,x}$	3.63	±	$0.18^{a;\,x}$	4.20	±	$0.23^{bc;\,x}$	5.99	±	$0.27^{c;\,w}$	5.48	±	$0.13^{bc;\,w}$
UL4	2.99	±	$0.10^{c;\,y}$	3.15	±	$0.09^{abc;\,y}$	3.78	±	$0.03^{cd;\,x}$	6.14	±	$0.31^{bc;\,w}$	6.26	±	$0.23^{a;\,w}$
TL1	4.00	±	$0.20^{a;\,yz}$	3.59	±	$0.28^{a;\,z}$	4.72	±	$0.32^{ab;\,xy}$	6.62	±	$0.29^{abc;\,w}$	5.19	±	$0.24^{bc;\,x}$
RS5	3.59	±	$0.30^{ab;\,y}$	3.34	±	$0.28^{ab;\,y}$	3.23	±	$0.38^{de;\,y}$	7.33	±	$0.24^{a;\,w}$	5.44	±	$0.25^{bc;\,x}$
RI11	3.62	±	$0.14^{ab;\,y}$	3.16	±	$0.19^{abc;\,y}$	3.84	±	$0.31^{cd;\,y}$	6.83	±	$0.22^{ab;\,w}$	5.70	±	$0.27^{ab;\,x}$
RG11	3.05	±	$0.11^{c;\,x}$	2.78	±	$0.05^{bc;\,x}$	2.95	±	$0.09^{e;\,x}$	6.70	±	$0.25^{abc;\,w}$	6.24	±	$0.33^{a;\,w}$
RG14	2.98	±	$0.08^{c;\,y}$	2.70	±	$0.07^{c;\,y}$	3.23	±	$0.20^{de;\,y}$	6.15	±	$0.22^{bc;\,w}$	5.31	±	$0.29^{bc;\,x}$
Combination	3.27	±	$0.14^{bc;\,y}$	3.34	±	$0.17^{abc;\,y}$	4.48	±	$0.31^{bc;\,x}$	6.71	±	$0.21^{abc;\,w}$	4.86	±	$0.28^{c;\,x}$

Notes:

- Values are mean ± standard error mean (SEM), n = 8.

- ^{abcde} Within a column, values with different superscripts are significantly different at $P < 0.05$

- ^{wxyz} Within a row, values with different superscripts are significantly different at $P < 0.05$

FIGURE 4: Effect of direct fed of metabolites on population of *Enterobacteriaceae* bacteria in different intestinal regions.

Metabolites	Population of *Enterobacteriaceae* bacteria (Log_{10} CFU/mL) in different intestinal regions														
	Duodenum			Jejunum			Ileum			Caecum			Colon		
Control	3.18	±	$0.13^{bc;z}$	3.29	±	$0.10^{abj;z}$	3.70	±	$0.20^{bc;y}$	5.19	±	$0.09^{cd;x}$	6.78	±	$0.13^{abc;w}$
UL4	3.18	±	$0.18^{bc;yz}$	2.70	±	$0.13^{c;z}$	3.78	±	$0.22^{bc;y}$	2.28	±	$0.32^{cd;x}$	7.57	±	$0.23^{a;w}$
TL1	3.19	±	$0.13^{bc;z}$	2.89	±	$0.23^{bc;z}$	3.82	±	$0.22^{bc;y}$	5.09	±	$0.11^{d;x}$	6.50	±	$0.24^{bc;w}$
RS5	3.41	±	$0.13^{ab;y}$	2.81	±	$0.12^{c;z}$	3.36	±	$0.16^{cd;y}$	5.75	±	$0.15^{abc;x}$	7.05	±	$0.25^{ab;w}$
RI11	3.68	±	$0.10^{a;y}$	2.89	±	$0.12^{bc;z}$	3.20	±	$0.14^{cd;z}$	6.09	±	$0.13^{ab;x}$	7.00	±	$0.27^{ab;w}$
RG11	2.60	±	$0.14^{d;y}$	2.88	±	$0.14^{bc;y}$	2.90	±	$0.20^{d;y}$	6.33	±	$0.23^{a;x}$	7.54	±	$0.33^{a;w}$
RG14	2.94	±	$0.15^{cd;y}$	2.84	±	$0.08^{bc;y}$	2.97	±	$0.18^{d;y}$	5.52	±	$0.21^{bcd;x}$	6.61	±	$0.29^{bc;w}$
Combination	3.11	±	$0.08^{bc;z}$	3.67	±	$0.09^{a;z}$	4.87	±	$0.32^{a;y}$	5.56	±	$0.17^{bcd;x}$	6.16	±	$0.28^{c;w}$

Notes:

- Values are mean ± standard error mean (SEM), n = 8
- ^{abcd} Within a column, values with different superscripts are significantly different at $P < 0.05$
- ^{wxyz} Within a row, values with different superscripts are significantly different at $P < 0.05$

FIGURE 5: Effect of direct fed of metabolites on population of lactic acid bacteria and *Enterobacteriaceae* bacteria in intestinal digesta.

Notes:- Values are mean ± standard error mean (SEM), n = 8.

- [ab] Within a column, values with different superscripts are significantly different at $P < 0.05$

Metabolites	Population of bacteria (Log_{10} CFU/mL) in intestinal digesta					
	Lactic acid bacteria			*Enterobacteriaceae* bacteria		
Control	4.74	±	0.30[a]	5.08	±	0.12[a]
UL4	4.21	±	0.13[ab]	5.32	±	0.23[a]
TL1	4.62	±	0.31[ab]	5.27	±	0.26[a]
RS5	4.54	±	0.36[ab]	5.08	±	0.16[a]
RI11	4.69	±	0.26[ab]	5.06	±	0.32[a]
RG11	4.12	±	0.12[ab]	5.09	±	0.22[a]
RG14	3.88	±	0.14[b]	5.31	±	0.29[a]
Combination	4.60	±	0.32[ab]	5.79	±	0.48[a]

Metabolites	Crypt depth (µm) of small and large intestinal regions														
	Duodenum			Jejunum			Ileum			Caecum*			Colon*		
Control	79.19	±	1.54$^{cd;x}$	76.53	±	1.35$^{de;x}$	68.84	±	1.13$^{d;y}$	149.44	±	3.85$^{f;w}$	182.47	±	2.50$^{b;v}$
UL4	77.31	±	1.01$^{d;w}$	77.19	±	1.39$^{de;w}$	76.09	±	1.18$^{bc;w}$	178.41	±	2.40$^{a;v}$	178.97	±	2.17$^{b;v}$
TL1	85.88	±	1.49$^{b;w}$	80.34	±	1.38$^{bcd;x}$	71.03	±	1.26$^{d;y}$	147.72	±	1.82$^{f;v}$	145.78	±	3.07$^{e;v}$
RS5	84.47	±	1.61$^{b;w}$	75.66	±	1.36$^{e;x}$	75.00	±	1.34$^{c;x}$	171.84	±	1.82$^{ab;v}$	170.34	±	2.99$^{c;v}$
RI11	82.66	±	1.60$^{bc;y}$	89.63	±	1.53$^{a;x}$	76.94	±	1.32$^{bc;z}$	152.56	±	2.47$^{ef;v}$	142.50	±	1.58$^{e;w}$
RG11	91.12	±	1.46$^{a;x}$	77.03	±	1.20$^{de;y}$	71.12	±	1.06$^{d;z}$	137.53	±	2.08$^{g;w}$	155.69	±	2.64$^{d;v}$
RG14	75.75	±	1.41$^{d;y}$	84.09	±	1.40$^{b;x}$	70.44	±	1.36$^{d;y}$	162.03	±	1.50$^{cd;w}$	179.19	±	4.06$^{b;v}$
Combination	86.97	±	1.61$^{b;x}$	79.00	±	1.35$^{cde;y}$	92.59	±	1.62$^{a;x}$	158.69	±	3.17$^{de;v}$	149.47	±	2.63d$^{e;w}$

FIGURE 6: Effect of direct fed of metabolites on crypt depth of different small and large intestinal regions

Notes:- Values are mean ± standard error mean (SEM), n = 8

- ^{abcdefg} Within a column, values with different superscripts are significantly different at P <0.05

- ^{vwxyz} Within a row, values with different superscripts are significantly different at P <0.05

-* represent large intestinal region

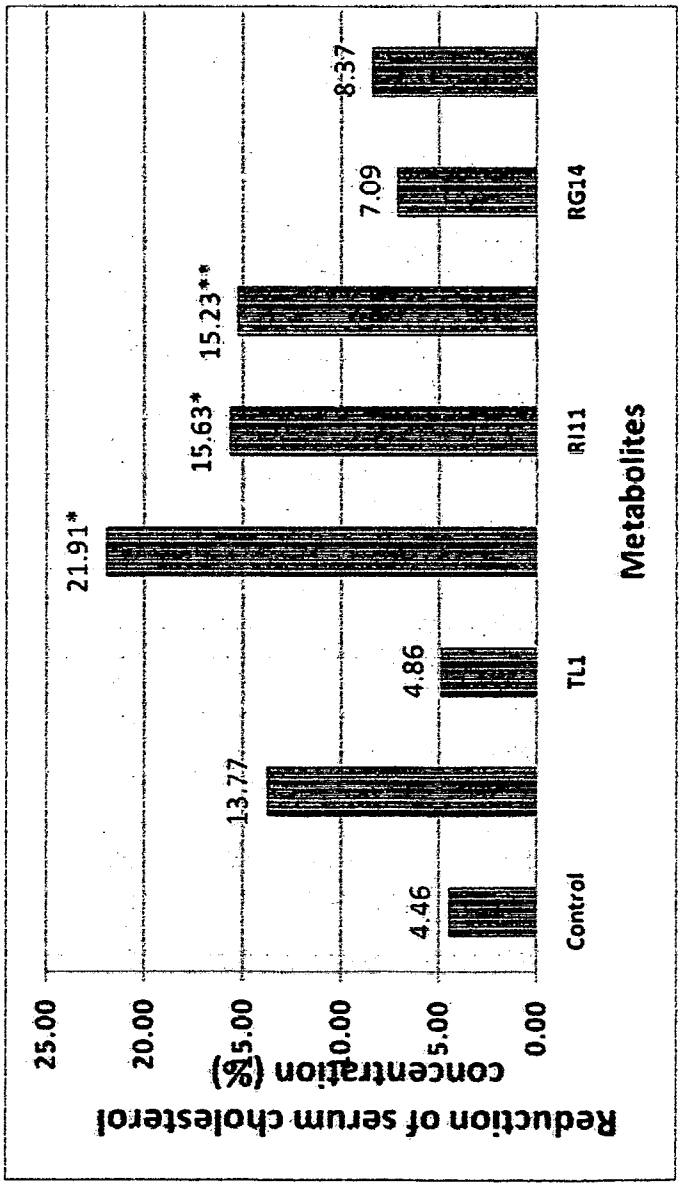
FIGURE 7: Effect of direct fed of metabolites on concentration of short chain fatty acids in Week-4 faecal samples

Metabolites	Concentration of SCFAs in faecal samples (mM/g of wet faeces)											
	Acetic acid			Propionic acid			Iso-Butyric acid			Butyric acid		
Control	48.91	±	8.86[a]	10.38	±	2.89[a]	0.21	±	0.07[a]	5.79	±	2.41[a]
UL4	49.88	±	8.41[a]	9.83	±	3.53[a]	0.22	±	0.02[a]	2.75	±	1.09[ab]
TL1	36.82	±	4.15[a]	5.54	±	0.94[a]	0.16	±	0.02[a]	1.68	±	0.46[b]
RS5	39.76	±	3.64[a]	7.26	±	1.11[a]	0.22	±	0.04[a]	2.06	±	0.80[ab]
RI11	46.48	±	9.12[a]	9.24	±	1.96[a]	0.18	±	0.05[a]	2.16	±	0.65[ab]
RG11	61.60	±	14.70[a]	14.54	±	4.75[a]	0.28	±	0.05[a]	3.84	±	1.13[ab]
RG14	52.00	±	11.10[a]	10.18	±	2.73[a]	0.28	±	0.07[a]	3.05	±	0.99[ab]
Combination	41.30	±	10.90[a]	8.31	±	2.16[a]	0.31	±	0.14[a]	2.72	±	0.99[ab]

- Values are mean ± standard error mean (SEM), n = 6.

- [ab] Within a column, values with different superscripts are significantly different at $P < 0.05$

FIGURE 8: Effect of direct fed of metabolites on serum cholesterol level of rats after feeding trial.



Notes:

- Values with asterisk are significantly different when compare with Week-0, * p<0.05; ** p<0.01

INTERNATIONAL SEARCH REPORT

International application No.
PCT/MY2010/000306

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

A61K 35/74 (2006.01) ***A61P 1/00*** (2006.01) ***C12N 1/20*** (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Files WPI, Medline, Epodoc, Caplus and Biosis keywords: Lactobacil?, probiotic#, Plantarum, I_UL4, TL1, RS5, RI11, RG11, RG14,bacteriocin, formic, lactic, organic, vitamin#, vancomycin,enterococ?, listeria, cereus, streptococ?, cholesterol and like terms

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	Foo, H.L. et al., "Effects of Adding *Lactobacillus plantarum* I-UL4 Metabolites in Drinking Water of Rats", Pakistan Journal of Nutrition, (2003), Vol. 2, No. 5, pages 283-288 See Pg. 285 last paragraph lines 1-5 and 18-23; Pg. 286 last paragraph; Pg.287 2nd paragraph and Abstract	1-9 11
X Y	Thanh N. T. et al., "Effects of feeding metabolite combinations produced by *Lactobacillus plantarum* on growth performance, faecal microbial population, small intestine villus height and faecal volatile fatty acids in broilers", British Poultry Science, (May 2009), Vol. 50, No. 3, pages 298-306 See Abstract; Pg. 299 right column 1st paragraph; Pg. 301 lines 19-30; Pg. 304 last paragraph; Pg. 303 right column lines 2-18; Pg. 302 and 304 second paragraphs; Pg. 302 right column second paragraph	1, 4-8, 10 & 12 11

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 March 2011	8 APR 2011
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. +61 2 6283 7999	Authorized officer **ARATI SARDANA** AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No : +61 2 6283 2905

INTERNATIONAL SEARCH REPORT

International application No.
PCT/MY2010/000306

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Foo H. L. et al., "Effects of Feeding *Lactobacillus plantarum* I-UL4 Isolated from Malaysian Tempeh on Growth Performance, Faecal Flora and Lactic Acid Bacteria and Plasma Cholesterol Concentrations in Postweaning Rats", Food Science and Biotechnology, (2003), Vol. 12, No. 4, pages 403-408 See Abstract; Pg. 406 last 3 lines; Pg. 405 second paragraph lines 12-23.	1, 4-9
P,X	WO 2010/117255 A1 (UNIVERSITI PUTRA MALAYSIA (UPM)) 14 October 2010 See Pg. 4 last two lines; Pg. 12 lines 1-3; claims 16 and 17	1, 4-10 and 12
E	WO 2011/019264 A1 (UNIVERSITI PUTRA MALAYSIA (UPM)) 17 February 2011 Pg. 4 lines 10-15; claims 1, 2 and 3; Pg. 9 lines 23-26; Pg. 11 lines 1-9; Pg. 10 lines 1-5; claim 23	1 and 4-8
P,X	Loh T.C. et al., "Feeding of different levels of metabolite combinations produced by *Lactobacillus plantarum* on growth performance, faecal microflora, volatile fatty acids and villi height in broilers", Animal Science Journal, (April 2010), Vol. 81, No. 2, pages 205-214 See Abstract	1, 4-8, 10 and 12
X	Loh T. C. et. al., "Effects on growth performance, faecal microflora and plasma cholesterol after supplementation of spray-dried metabolite to postweaning rats", Czech Journal of Animal Science, (January 2009), Vol. 54, No. 1, pages 10-16 See Abstract	1 and 4-9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/MY2010/000306

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:

   a. (means)

   ☐ on paper

   ☐ in electronic form

   b. (time)

   ☐ in the international application as filed

   ☐ together with the international application in electronic form

   ☐ subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

Sequence listing furnished on paper and in an electronic form with the international application as filed was not used for the purpose of this search and opinion.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/MY2010/000306

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member			
WO	2010117255	AU	2009344224	MY	2009000050
WO	2011019264	NONE			

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

END OF ANNEX