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SHORT COMMUNICATION

Lactobacilli possess inhibitory activity against dipeptidyl peptidase-4 (DPP-4)

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Abstract Dipeptidyl peptidase-4 (DPP-4) plays an important role in the enzymatic inactivation of incretin hormones. In this context, drugs that inhibit DPP-4 have been developed and clinically approved as therapies for type 2 diabetes. As the primary substrates of DPP-4 are produced in the intestinal lining, we investigated whether lactobacilli colonizing the gut could inhibit this enzyme. Fifteen *Lactobacillus* strains (Lb 1–15) from human infant faecal samples were isolated, identified, extracted and screened for inhibitory activity against DPP-4. Activity was compared against *Lactobacillus* reference strains (Ref 1–7), a Gram-positive control (Ctrl 1) and two Gram-negative controls (Ctrl 2–3). A range of DPP-4 inhibitory activity was observed (10–32 %; $p<0.05$–0.001). Strains of *L. plantarum* (12–25 %) and *L. fermentum* (14 %) had significant inhibitory activity. However, we noted that *Escherichia coli* (Ctrl 2) and *Salmonella* Typhimurium (Ctrl 3) had the greatest inhibitory activity (30–32 %). Contrastingly, some isolates (Lb 12–15) and reference cultures (Ref 1–4), instead of inhibiting DPP-4, actually enhanced it, perhaps indicating the presence of X-prolyl-dipeptidyl-amino-peptidase (PepX). This provides a future rationale for using probiotic bacteria or their components for management of type 2 diabetes via DPP-4 inhibition.

Keywords Amino-methyl-coumarin · Dipeptidyl peptidase-4 · Glucagon-like peptide-1 · Glucose-dependent insulinotropic peptide · *Lactobacillus* · Lactic acid bacteria

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Dipeptidyl peptidase-4 (DPP-4) is a physiological enzyme found both membrane-bound and circulating in the blood, and one of its primary functions is the inactivation of the incretin hormones glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) (Flatt et al. 2008). Inhibition of DPP-4 prolongs the circulating half-life of endogenous incretin hormones, enhancing their insulinotropic and glucoregulatory activity. For this reason, DPP-4 inhibitors were proposed as a new therapeutic strategy for type 2 diabetes, and efforts by the pharmaceutical industry have led to the development, launch and clinical use of DPP-4 inhibitor drugs (also referred to as gliptins) (Green et al. 2006a,b). Gliptins appear to be safe, efficacious and generally well tolerated, achieving glucose homeostasis without the typical associated risk of hypoglycaemia or increased weight gain (Deacon and Holst 2013). In addition, under some circumstances, DPP-4 inhibition appears to improve cardiovascular risk factors, reduce blood pressure, improve postprandial hyperglycaemia, reduce inflammatory markers, diminish oxidative stress, improve endothelial function, and reduce platelet aggregation in patients with type 2 diabetes mellitus (Scheen 2013). Despite the wide range of available anti-diabetic drugs, considerable unmet medical needs still exist. The discovery and production of new pharmaceutical agents is expensive, and a growing number of studies are investigating natural

sources of DPP-4 inhibitory activity, which could offer safe and cost-effective alternative treatment strategies.

Amino acids and dipeptides from food proteins have recently been explored and were reported to inhibit DPP-4 (Nongonierma and Fitzgerald 2013). Dietary proteins including casein (cow's milk) and collagen (bovine meat and salmon) have been suggested as the richest sources of potential DPP-4 inhibitors (Lacroix and Li-Chan 2012). Several dietary peptides mimic terminal dipeptides that are hydrolyzed by DPP-4 and serve as competitive inhibitors for the DPP-4 enzyme (Lacroix and Li-Chan 2012). Similarly, milk protein hydrolysates have been evaluated for DPP-4 inhibitory activity, and five milk protein hydrolysates were shown to competitively inhibit DPP-4 (Nongonierma and Fitzgerald 2013). Interestingly, dose-dependent inhibition of DPP-4 was recently demonstrated by peptides (>1422 Da) isolated from tuna cooking juice, which can actively pass through the digestive tract, retaining inhibitory potential (Huang et al. 2008). A small number of studies have reported medicinal plant DPP-4 inhibitory activity (Lendeckel et al. 2002; Parmar et al. 2012). Thus far, however, no published studies have determined whether probiotic bacterial cultures possess or produce DPP-4 inhibitory activity. The purpose of this investigation was to screen a range of heat-killed sonicated extracts of *Lactobacillus* spp. for DPP-4 inhibitory activity.

The application of lactic acid bacteria such as *Lactobacillus* spp. is a novel potential lifestyle intervention for alleviating the symptoms of type 2 diabetes mellitus, and in the future could act as an adjunct to diabetes treatment (Panwar et al. 2013, 2014). Some lactic acid bacteria have recognized anti-inflammatory effects on the intestine and are used in clinical practice (Ritchie and Romanuk 2012). VSL#3 (VSL Pharmaceuticals, Inc., Gaithersburg, MD, USA), for example, is a probiotic bacterial preparation classified by the FDA as a 'medicinal food' that may be useful in the dietary management of three major gastrointestinal conditions: ulcerative colitis, ileal pouchitis and irritable bowel syndrome (Chapman et al. 2007). Probiotics such as these have good safety and tolerability profiles, and side effects are uncommon (Chapman et al. 2007). Studies are needed to scientifically investigate and characterize the potential anti-diabetic activity of probiotic bacteria, as they could potentially play an important role in providing adjuncts to existing therapies and in new preventive or prophylactic strategies, or may lead to the discovery of new pharmacological compounds.

This study profiled various *Lactobacillus* strains for DPP-4 inhibitory activity, including strains isolated from infant faecal samples and bacterial reference cultures (Table 1). In brief, faecal samples were collected from five healthy breastfed infants younger than 9 months of age, living in Shamli, Uttar Pradesh, India. In each case, full parental consent was obtained. Faecal samples were collected in sterile containers with pre-sterilized swabs. Samples were transferred to the laboratory and pre-incubated in MRS (M369, HiMedia Laboratories, Mumbai, India) broth tubes, serially diluted and plated over MRS agar plates. Plates were incubated overnight at 37 °C for development of colonies. Individual colony-forming units were picked, purified and identified morphologically on the basis of Gram's staining. The identity of Gram-positive, catalase-negative *Lactobacillus* rods was further ascertained genotypically by genus-specific PCR and 16S rRNA sequencing (Panwar et al. 2014). Given that *L. plantarum* is typically not the most abundant *Lactobacillus* species found in infant faeces, it was surprising that the majority of viable isolates were from this species. Intra-individual day-to-day variability and inter-individual variability of microorganisms were not assessed. The study was based on the rationale that since the intestine is the primary site of incretin hormone secretion (enteroendocrine cells are found in the intestinal lining), gut bacteria such as lactobacilli and their secretions will come into close proximity with incretin hormones and, upon absorption into the bloodstream, could accompany and protect them from DPP-4. Also, many bioactive components/metabolites produced by intestinal probiotic bacteria have been demonstrated to cross the intestinal membrane and enter the blood circulation (Selkrig et al. 2014). Therefore, establishing the presence of DPP-4 inhibitory activity in gut microbiota will generate new lines of enquiry concerning their anti-diabetic potential.

Overnight broth cultures were harvested and bacterial pellets washed twice in 1X PBS [phosphate-buffered saline] (12, 000 *g*; 15 min), followed by re-suspension in nuclease-free water on a weight/volume basis. Bacterial pellets of 500 mg (wet weight) were re-suspended in 1 ml nuclease-free water and mixed by pipetting and vortexing. Bacterial suspensions were heat killed (65 °C; 30 min) in a water bath, sonicated (20 kHz, 3×30 s pulse) and stored at −80 °C until assayed for DPP-4 inhibitory activity. DPP-4 activity was determined fluorometrically using the method of Fujiwara and Tsuru (1978), which measures the amount of free AMC (7-amino-4-methyl-coumarin) liberated from the DPP-4 substrate, Gly-Pro-AMC (Sigma-Aldrich Co. Ltd., Dorset, UK). Assays were conducted in triplicate in 96-well microtitre plates, with fluorescence emission measured at Em430 nm following excitation at Ex351 nm using a Tecan Safire desktop fluorometer (Tecan UK Ltd., Theale, Reading, UK). Test samples (50 μl) were analysed in triplicate in 96-well microtitre plates containing Gly-Pro-AMC. Negative-control wells contained PBS buffer (50 μl) and Gly-Pro-AMC (1 mM). The reaction was initiated by the addition of DPP-4 (1 U/ml, Calbiochem; Merck Millipore, Nottingham, UK), and plates were incubated at 37 °C with gentle agitation for 1 h, after which 100 μl of 3 mM acetic acid was added to terminate reactions. Berberine (13 mM; Sigma-Aldrich Co.), a previously reported plant compound with DPP-4 inhibitory activity (Al-Masri et al.

Table 1 Test strains examined in this study

No.	Strain	Type	Species	Accession No./Gene Bank No.
1	Lb1	Faecal isolate	*Lactobacillus plantarum*	Unknown
2	Lb2	Faecal isolate	*L. plantarum*	Unknown
3	Lb3	Faecal isolate	*L. plantarum* subsp. *argentorotensis*	KC491380
4	Lb4	Faecal isolate	*L. plantarum*	KF678450
5	Lb5	Faecal isolate	*L. plantarum*	Unknown
6	Lb6	Faecal isolate	*L. plantarum*	Unknown
7	Lb7	Faecal isolate	*L. fermentum*	Unknown
8	Lb8	Faecal isolate	*L. plantarum*	KF678451
9	Lb9	Faecal isolate	*L. plantarum*	KF678452
10	Lb10	Faecal isolate	*L. plantarum*	KF678453
11	Lb11	Faecal isolate	*L. plantarum*	Unknown
12	Lb12	Faecal isolate	*Lactobacillus* sp.	Unknown
13	Lb13	Faecal isolate	*L. fermentum*	KC866340
14	Lb14	Faecal isolate	*Lactobacillus plantarum*	Unknown
15	Lb15	Faecal isolate	*L. acidophilus*	Unknown
16	Ref1	Reference culture	*L. acidophilus*	NCIMB 701748
17	Ref2	Reference culture	*L. casei*	NCIMB 4114
18	Ref3	Reference culture	*L. fermentum*	NCIMB 2797
19	Ref4	Reference culture	*L. johnsonii*	NCIMB 8795
20	Ref5	Reference culture	*L. paracasei*	NCIMB 1407
21	Ref6	Reference culture	*L. plantarum*	NCIMB 1406
22	Ref7	Reference culture	*L. rhamnosus*	NCIMB 6375
23	Ctrl1	Gram-positive control	*Bifidobacterium bifidum*	NCIMB 702715
24	Ctrl2	Gram-negative control	*Escherichia coli* K12	NCTC 10538
25	Ctrl3	Gram-negative control	*Salmonella* Typhimurium	Unknown

2009), was used in each experiment as a positive control. Data were expressed as percentages (mean ± SEM) and compared with controls by means of one-way ANOVA using the Dunnett post hoc test.

In this study, DPP-4 inhibitory activity of various lactobacilli was examined and compared against some Gram-negative and Gram-positive controls. Tests were carried out with heat-killed sonicated extracts of bacteria to ensure that any inhibitory activity observed was not the result of bacterial metabolism/fermentation. The positive control berberine produced significant DPP-4 inhibition, reaching 75 % ($p<0.00,1$) and it is believed that this is at least partially responsible for its previously reported in vivo anti-hyperglycaemic action (Al-Masri et al. 2009). Among the *Lactobacillus* extracts tested, the greatest level of DPP-4 inhibition was demonstrated by strains Lb 1–4 (25, 20, 24 and 22 % respectively ($p<0.001$). Other *Lactobacillus* extracts, Lb 5, 6, 9, 10 (*L. plantarum*) and Lb 7 (*L. fermentum*), significantly ($p<0.05$–0.01, $n=3$) inhibited DPP-4 but to a lesser extent (10–14 %, respectively). Of the reference *Lactobacillus* cultures studied, only *L. paracasei* showed DPP-4 inhibitory potential, of around 25 % ($p<0.001$, $n=3$). In contrast, some of the *Lactobacillus* isolates (Lb 12–15) and reference strains (Ref 1, 3–4; *L. acidophilus, L. fermentum, L. johnsonii*) potentiated the enzymatic activity of DPP-4. The presence of these heat-killed sonicated bacteria enhanced the activity of DPP-4, resulting in the release of significantly more free AMC than by controls, as indicated by negative values (Fig. 1). Other isolates (Lb 8 and 11), Reference probiotic strains (Ref 2, 6–7; *L. casei, L. plantarum*, *L. rhamnosus*) and *Bifidobacterium bifidum* (Ctrl 1) demonstrated no inhibitory or stimulatory effects.

It is particularly interesting that the Gram-negative control bacteria *Escherichia coli* K12 (Ctrl 2) and *Salmonella* Typhimurium (Ctrl 3), tested in parallel with the *Lactobacillus* spp., exhibited the strongest DPP-4 inhibitory activity ($p<0.001$, $n=3$; 32 and 30 %, respectively). These bacteria would not be regarded as 'probiotic' in nature, and were chosen simply because they were available within our laboratory. However, we are aware of *E. coli* strains being investigated as potential probiotics (Huebner et al. 2011), so perhaps this area is worthy of further investigation.

This study has focused on whether DPP-4 inhibitory activity is present in bacteria commonly colonizing the human intestine. To the best of our knowledge, DPP-4 inhibitory

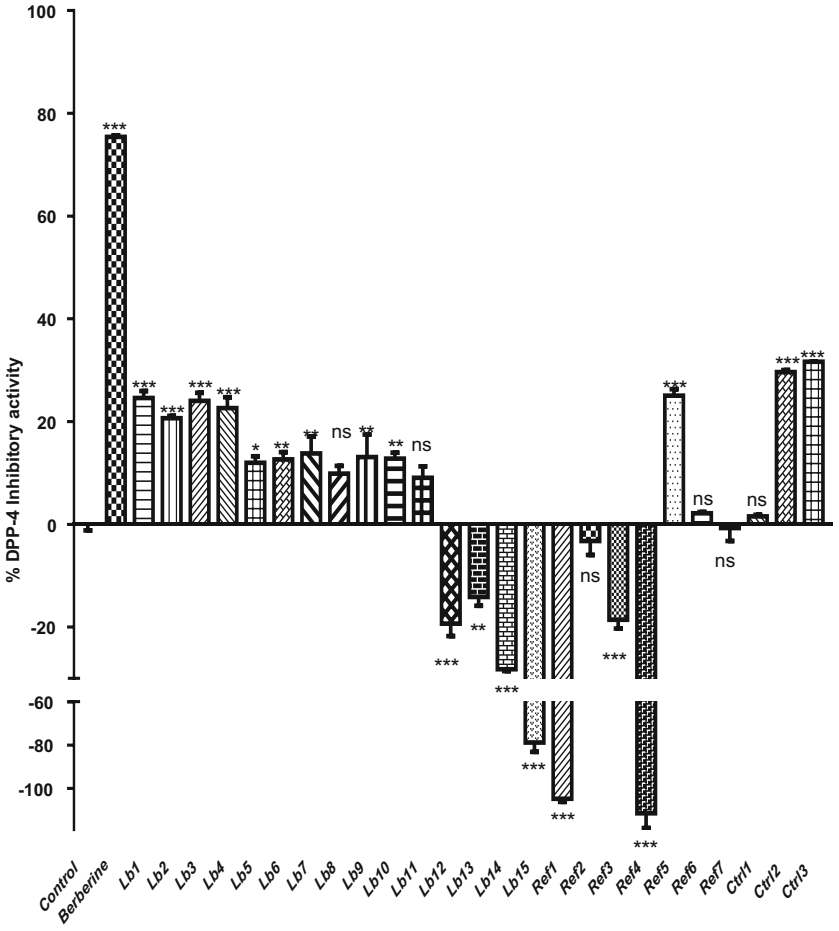

Fig. 1 DPP-4 inhibitory activity of lactobacilli and other bacteria. The figure shows the percentage DPP-4 inhibitory activity demonstrated by heat-killed sonicated extracts of *Lactobacillus* strains (Lb 1–15) isolated from human infant faecal samples, *Lactobacillus* reference strains (Ref 1–7), a Gram-positive control (*Bifidobacterium bifidum*, Ctrl 1) and two Gram-negative controls (*Escherichia coli* K12 - Ctrl 2 and *Salmonella* Typhimurium - Ctrl 3). Negative values indicate the presence of DPP-4-like activity. Data are expressed as mean ± SEM. *$p<0.05$, **$p<0.01$ and ***$p<0.001$ compared with vehicle control. ns = not statistically significant

activity has not previously been reported for *Lactobacillus* or any other species of lactic acid bacteria. However, we do acknowledge reports of activity in other bacterial cultures, including *Bacillus* and *Streptomyces* spp. Diprotin A (one of the earliest known DPP-4 inhibitors) and diprotin B have been isolated from culture filtrates of Gram-positive *Bacillus cereus* BMF673-RF1 (Umezawa 1984). Similarly, a novel DPP-4 inhibitor, sulphostin, was isolated from the culture broth of *Streptomyces* sp. MK251-43F3 (Abe et al. 2005).

Our results demonstrate that some *Lactobacillus* strains (Lb 1–4) and a reference culture of *L. paracasei* (Ref 5) possessed a reasonable amount of DPP-4 inhibitory activity. Extracts of a number of other isolates exhibited some level of inhibitory activity (Lb 5–11), whereas other species of *Lactobacillus* (Ref 6–7) were largely devoid of activity, as was one species of *Bifidobacterium* (Ctrl 1). We also discovered that a number of *Lactobacillus* isolates (Lb 12–15) and reference cultures (Ref 1–4), instead of inhibiting DPP-4, actually promoted the hydrolysis of the Gly-Pro-AMC substrate, therefore indicating the presence of DPP-4-like activity in these bacteria. Although this finding was initially surprising, and it prompted us to recheck and repeat our studies, a thorough search of the literature provided a straightforward and rational explanation. DPP-4-like activity in bacteria appears to be the result of a bacterial enzyme called X-prolyl-dipeptidyl-amino-peptidase (PepX). PepX is a proline-specific peptidase with enzymatic activity almost identical to that of DPP-4, i.e., removal of N-terminal dipeptide residues from peptides containing a proline in the penultimate position (Meyer-Barton et al. 1993). The PepX gene or PepX activity have been reported on a few occasions in lactic acid bacteria, including *L. acidophilus* (Bockelmann and Fobker 1991), *L. casei* (Habibi-Najafi and Lee 1994), *L. curvatus* DPC2024 (Magboul and McSweeney 2000), *L. sanfranciscensis, L. lactis*, *L. delbrueckii*, *L. helveticus*, *L. rhamnosus* and *Streptococcus thermophilus* (Savijoki et al. 2006). The existence of an enzyme such as PepX provides a possible explanation as to why DPP-4 inhibitory activity can be found in bacteria, in that it may be produced to regulate enzymatic activity or bacterial metabolism, or to help the bacterium compete with other bacterial species.

In conclusion, this study reports that some strains of *Lactobacillus* (*L. plantarum* and *L. fermentum*) as well as *Salmonella* Typhimurium and *E. coli* are potential sources of DPP-4 inhibitory activity, and there may be opportunities in the future to use probiotic bacteria in the management of type

2 diabetes. It is still unclear which bacterial metabolites are responsible and whether they are absorbed across the intestinal membrane into the circulation. Further work is needed to identify the responsible molecules and to better understand the variations in inhibitory and PepX activity between strains.

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