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RESEARCH PAPER

Probiotic Treatment Proceeded by a Single Dose of Bile Acid and Gliclazide Exert the Most Hypoglycemic Effect in Type 1 Diabetic Rats

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Abstract. Probiotics have beneficial effects in treating diabetes and may form a useful adjunct to established hypoglycemic drugs. Here we report the effect of pre-treatment with probiotics followed by the oral administration of gliclazide + MKC (a semisynthetic bile acid), on blood glucose levels (BGL) and gliclazide pharmacokinetics in a rat model of type 1 diabetes (T1D). Male Wister rats were randomly divided into 8 groups (N = 10), 4 of which were made diabetic by i.v. injection of alloxan. Groups 1–4 were administered a single dose of gliclazide + MKC (20 and 4 mg/kg, respectively) by oral gavage while groups 5–8 by i.v. Group 1 was healthy while group 2 was diabetic. Groups 3 (healthy) and 4 (diabetic) were gavaged with probiotic mixture for three days and 12 h after the last ingestion of probiotics, a baseline blood sample was taken from all 8 groups of rats and gliclazide + MKC was administered. Blood samples collected prior to gliclazide revealed probiotic treatment significantly reduced BGL in diabetic rats which were further reduced after gliclazide + MKC oral dose. Moreover, in probiotic treated healthy rats, gliclazide bioavailability was the lowest. In contrast, in probiotic treated diabetic rats, gliclazide bioavailability was higher than untreated diabetic rats. Probiotic treatment lowers BGL and increases gliclazide absorption in this model of T1D. Our results suggest that a multidrug approach to treating diabetes can prove useful with MKC, gliclazide and probiotics being potential adjuvant treatments.

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Abbreviations used: T1D, type 1 diabetes; BGL, blood glucose level.

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1. Introduction

Type 1 diabetes (T1D) is a common disease that predominantly develops during childhood. The clinical presentation of T1D is marked hyperglycaemia [1] which result from the destruction of the pancreatic β cells [2,3]. Although a number of hypotheses explaining the pathogenesis of T1D [3] have been proposed, the most accepted explanation is that T1D is a chronic inflammatory disease which is triggered in genetically susceptible individuals by the dysfunction of the gut-mediated oral tolerance to ingested proteins and the consequent change in the bile acids metabolism [4-6].

MKC is a semisynthetic analogue of cholic acid [7] and has a hypoglycemic effect when given orally to type 1 diabetic rats [8]. Furthermore, a stronger hypoglycemic effect was noticed in diabetic rats following the oral administration of the combined mixture of MKC and the antidiabetic drug gliclazide [9]. Gliclazide is a second generation sulphonylurea used to treat type 2 diabetes [10,11]. Its primary mode of action is to induce insulin secretion by pancreatic β-cells [12,13]. Gliclazide has also other beneficial effects such as stimulating the synthesis of glucose transporters [14], reducing hepatic glucose production, improving glucose turnover, and improving glucose uptake by muscles [15,16]. Accordingly, its application in type one diabetes can prove useful especially when combined with other hypoglycemic agents [9,17]. In an in vivo study in our laboratory, we have shown that probiotic treatment for three days resulted in a hypoglycemic effect on alloxan-induced diabetic rats [18].

Probiotics are dietary supplements that contain live bacteria, which when administered in adequate amounts, confer a health benefit on the host (19). In order to achieve a synergistic effect, combinations of different bacterial strains should be used in probiotics [20,21] with *Lactobacillus* and *Bifidobacteria* being a common choice [21]. In one in vivo study, probiotics induced the production of IL-10 which has been linked with the prevention of T1D [22]. Moreover, in studies using probiotics, researchers were able to show clinical improvements of patients with T1D as well as certain degree of prevention if administered early [5,23-27].

With probiotics and bile acids presenting a great potential for T1D treatment, their integration with other antidiabetic drugs, in a multi-drug therapy should be investigated thoroughly. The aim of this in vivo study was to investigate the influence of MKC as a hypoglycemic agent, on the bioavailability of the antidiabetic drug gliclazide in diabetic rats treated with probiotics.

2. Materials and Methods

2.1. Materials

Gliclazide (99.9%) and alloxan (98%) were purchased from Sigma Chemical Co, St Louis, MO, USA. Ultra water-soluble transmission gel (hypoallergenic) was purchased from Medtel Pty. Ltd. NSW, Australia. MKC was synthesized and purified in the Department of Pharmacology, University of Novi Sad, Serbia, by the method of Miljkovic et al. [28]. Freeze-dried cultures of *Lactobacillus acidophilus, Bifidobacterium lactis,* and *Lactobacillus rhamnosus* were kindly provided by Professor John Tagg (Department of Microbiology, University of Otago, New Zealand) and Dr. Chris Chilcott (BLIS Technologies, Dunedin, New Zealand). The cultures were originally HowaruTM strains obtained from Danisco NZ Ltd, Auckland, New Zealand. All other chemicals and solvents were of HPLC grade.

2.2. Drugs preparations

A gliclazide suspension (20 mg/mL) was prepared by adding gliclazide powder to 10% Ultra water-soluble gel and while a MKC solution (5 mg/mL) was prepared by adding MKC powder to 2% $NaHCO_3$ solution. Drug preparations were mixed thoroughly at 37°C for 6 h, stored in the refrigerator, and were used within 48 h of preparation. A probiotic suspension (75 mg/kg, 1011 cells/g) containing equal amounts of *Lactobacillus acidophilus, Bifidobacterium lactis,* and *Lactobacillus rhamnosus*, was prepared by mixing the probiotic powders with HPLC water and was used immediately after preparation.

2.3. Animals

Male Wister rats (age 2–3 months, weight 350 ± 50 g) were maintained in an experimental animal facility and given standard diet and water ad libitum. Temperature and light were controlled to mimic the natural habitat. Eighty rats were randomly divided into 8 groups (N = 10), four of which were made diabetic by injecting freshly prepared alloxan solution (30 mg/mL) intravenously into the tail vein at a dose of 30 mg/kg [17,29,30]. Rats with blood glucose > 20 mmol/L and serum insulin <0.04 μg/L 2 to 3 days after alloxan injection were considered diabetic [17,31-33]. Diabetic rats showed signs of polydipsia, polyuria, weight loss and asthenia [29,34]. The study was approved by the Otago University Animal Ethics Committee.

2.4. Experimental protocol

Groups 1–4 were administered a single dose of gliclazide + MKC (20 and 4 mg/kg, respectively) by the oral route (gavage) while groups 5-8 were administered drug(s) via the intravenous (i.v) route (tail vein). Group 1 was healthy while group 2 was made diabetic as described above. Groups 3 (healthy) and 4 (diabetic) were gavaged with probiotic mixture twice daily for three days then 12 h after the last ingestion of probiotics, a baseline blood sample was taken from all 8 groups of rats and a single dose of gliclazide + MKC was administered either via the oral route (groups 1-4) or i.v. route (groups 5–8). Blood samples were then collected from the tail vein at 5, 10, 20, 40, 60, 80, 120, 150, 180, 240, 360 and 600 min after the dose. Insulin concentration in blood was measured using Mercodia Ultra Sensitive Rat Insulin Elisa (Sweden). Blood glucose was measured using an ACCU-CHEK Glucose Advantage Meter (Roche). Serum was separated by centrifugation at 15000 rpm for 5 min and kept at –20°C pending analysis for gliclazide within 2 days of collection.

2.5. HPLC

Gliclazide concentration in serum was measured by HPLC using a method based on the literature [35,36]. Acetonitrile was added to serum samples in a 2:1 ratio and, after vortexing for 10 s and centrifuging (15000 rpm) for 15 min, the supernatant (20 μL) was injected into the HPLC system. This consisted of a Shimadzu, SIL-10AD VP autoinjector, a C_{18} guard column (4 × 2.0 mm, Phenomenex), a C_{18} analytical column (100 × 2.0 mm Luna 5 μm, Phenomenex) and a Shimadzu, UV-V15 detector set at 229 nm. The mobile phase was acetonitrile 49% and water 51%, pH 2.7 (adjusted using orthophosphoric acid) at a flow rate of 0.4 mL/min. Under these conditions, the retention time for gliclazide was 2.9 min. A gliclazide standard curve was constructed in serum in the range 0.5–100 μg/mL. The assay was linear with a within-day coefficient of variation ranging from 1.2% at 100 μg/mL to 2.9% at 0.5 μg/mL. The limit of detection was 0.5 μg/mL and the limit of quantitation was 0.8 μg/mL. The recovery of gliclazide from serum was 89 ± 4%.

2.6. Pharmacokinetic analysis

Pharmacokinetic (PK) and pharmacodynamic (PD) parameters were calculated from the serum concentration–time data using the WinNonLin, version 4.1 (SCI software, Pharsight Corporation, Gary, NC, USA) while statistical comparisons were made using Minitab (Minitab, Version 14 ; Minitab Inc, Pennsylvania, USA).

Gliclazide serum concentration–time data were analyzed using a noncompartmental model. Maximum concentration (C_{max}) and the time to maximum concentration (t_{max}) were derived directly from the data. Total area under the plasma concentration-time curve (AUC_{0-t}) was calculated by the trapezoidal method and extrapolated to infinity ($AUC_{0-\infty}$). Half-life ($t_{1/2}$) was calculated from the elimination rate constant ke (determined using the last three points). Bioavailability (F) was calculated as AUC_{0-t} (oral)/AUC_{0-t} (i.v.). The mean residence time (MRT) was calculated from the $AUC_{0-\infty}$. Total body clearance (Cl) was calculated as Dose/ AUC_{0-t}. The volume of distribution area (Vd_{area}) was calculated using the equation [37]:

$$Vd_{area} = \text{dose} / (AUC0\text{-}\infty \times \beta).$$

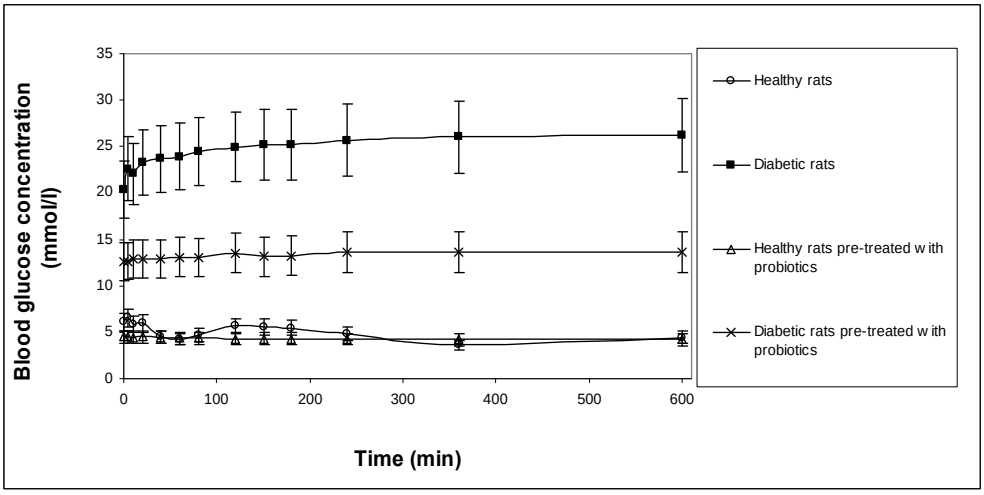

Figure 2. Blood glucose concentration-time curves after i.v. administration of gliclazide + MKC (20 and 4 mg/kg, respectively) to healthy and diabetic rats with and without probiotic pre-treatment.

2.7. Pharmacodynamic and PK/PD analysis

The effect of gliclazide + MKC on blood glucose concentration was calculated according to the sigmoid Emax model. The PK/PD equation was expressed as follows: $E = E_0 - \{(E_{max} \times C_e) / EC_{50} + C_e)\}$ in which E is the hypoglycemic effect of the drug, E_0 is the baseline blood glucose level before drug administration, EC_{50} is the concentration of drug producing 50% of the maximum effect on blood glucose, E_{max} is the maximum effect on blood glucose level resulting from drug administration, and C_e is the drug concentration in serum which produces the hypoglycemic effect (E). For C_e, E_{max}, E_o and EC_{50} measurements, PD model 108 was used [Inhibitory Effect Sigmoid E maximum, with $C_e = 0$ at E_o and $C_e = \infty$ at E_{max}].

2.8. Statistical analysis

Values are expressed as mean ± SD. Statistical

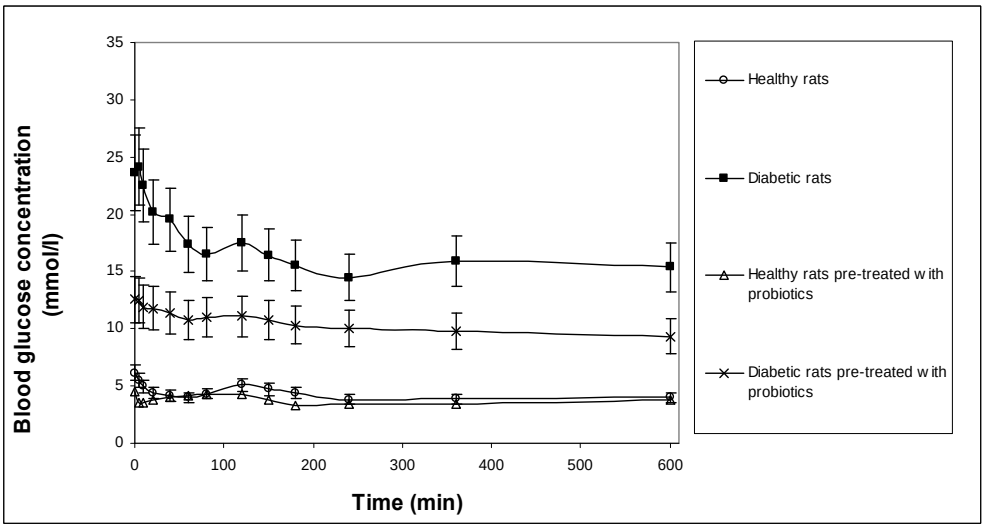

Figure 1. Blood glucose concentration-time curves after oral administration of gliclazide + MKC (20 and 4 mg/kg, respectively) to healthy and diabetic rats with and without probiotic pre-treatment.

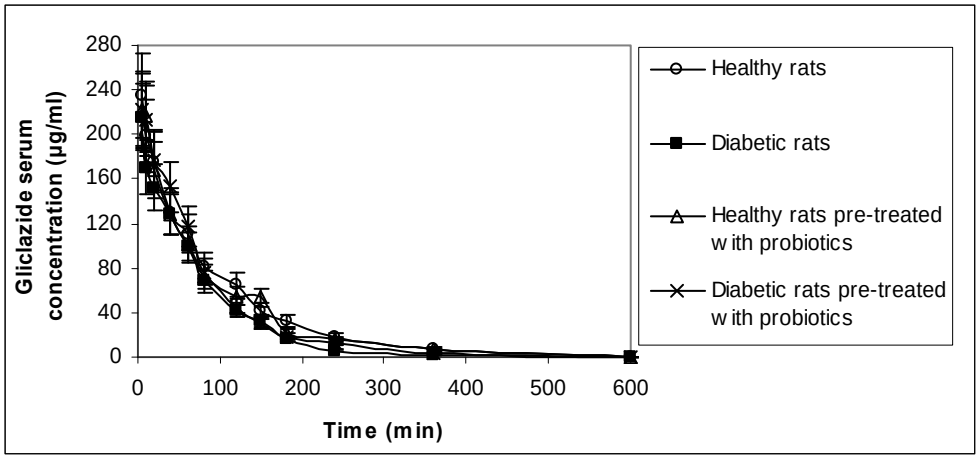

Figure 3. Gliclazide serum concentration after i.v. administration of gliclazide + MKC (20 and 4 mg/kg, respectively) to healthy and diabetic rats with and without probiotic pre-treatment.

comparisons between healthy and diabetic rats after oral and i.v. administration were carried out using the Wilcoxon matched pairs test at 95% CI while statistical comparisons between probiotic treated and untreated rats after oral and i.v. administration were carried out using the General Linear Model (GLM) to perform analysis of variance (ANOVA) with probiotic treatment as the discrete (fixed) factor at an α level of 0.05. Statistical significance was set at $P < 0.05$.

3. Results

Treatment with probiotics for three days had no effect on blood glucose levels in healthy rats but significantly reduced the elevated blood glucose levels in diabetic rats from 23.8 ± 3.0 mmol/L to 12.6 ± 2.0 mmol/L ($P < 0.01$). Furthermore, there were no significant changes in blood glucose in any group of rats after the i.v. (Fig. 1) but, after oral administration of MKC + gliclazide to untreated diabetic rats, the elevated

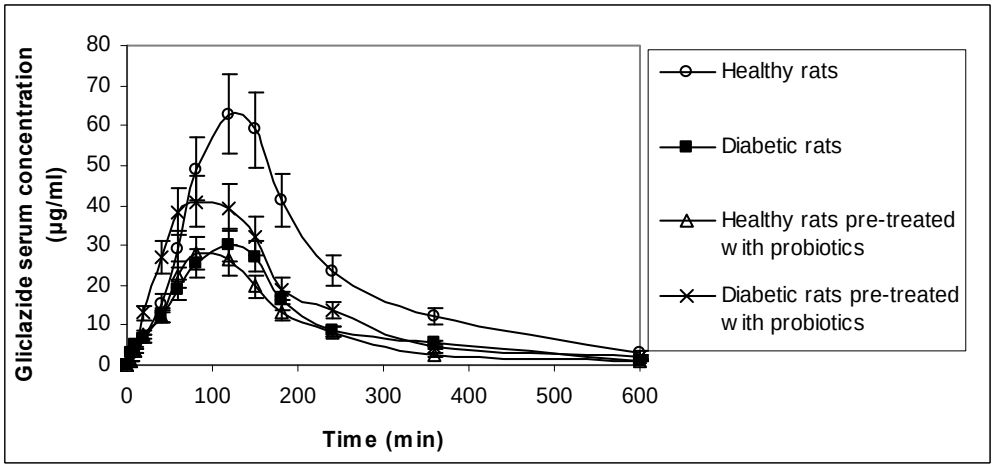

Figure 4. Gliclazide serum concentration after oral administration of gliclazide + MKC (20 and 4 mg/kg, respectively) to healthy and diabetic rats with and without probiotic pre-treatment.

TABLE 2. Pharmacokinetic parameters of gliclazide after i.v. administration of gliclazide + MKC (20 and 4 mg/kg, respectively) to healthy and diabetic rats with and without probiotic pre-treatment. Data are means ± SD (N = 10).

Pharmacokinetic parameters	i.v. healthy	i.v. diabetic	Sig.	i.v. healthy-probiotics	i.v. diabetic-probiotics	Sig.
MRT (min)	486 ± 124	470 ± 115	NS	451 ± 112	480 ± 183	NS
Vd_{area} (mL/kg)	127 ± 34	119 ± 44	NS	156 ± 40	146 ± 34	NS
T½ (min)	102 ± 39	100 ± 26	NS	113 ± 58	126 ± 52	NS
C_{max} (μg/mL)	-	-	-	-	-	-
t_{max} (min)	-	-	-	-	-	-
AUC_{0-t} (min × μg /mL)	(2.01 ± 0.57) x 10^4	(1.46 ± 0.66) x 10^4	NS	(1.91 ± 0.24) x 10^4	(1.98 ± 0.35) x 10^4	NS
$AUC_{0-\infty}$ (min × μg/mL)	(2.20 ± 0.74) x 10^4	(1.58 ± 0.58) x 10^4	NS	(1.99 ± 0.50) x 10^4	(2.08 ± 0.51) x 10^4	NS
Cl (μg /mL/min)	0.019 ± 0.008	0.021 ± 0.013	NS	0.026 ± 0.010	0.021 ± 0.011	NS

Sig: significance level
NS: not significant

TABLE 2. Pharmacokinetic parameters of gliclazide after i.v. and oral administration of gliclazide + MKC (20 and 4 mg/kg, respectively) to healthy and diabetic rats with and without probiotic pre-treatment. Data are means ± SD (N = 10).

Pharmacokinetic parameters	Oral healthy	Oral diabetic	Sig.	Oral healthy-probiotics	Oral diabetic-probiotics	Sig.
MRT (min)	484 ± 247	462 ± 197	NS	466 ± 139	462 ± 197	NS
Vd_{area} (mL/kg)	143 ± 30	138 ± 44	NS	138 ± 29	153 ± 65	NS
T½ (min)	123 ± 67	161 ± 42	NS	141 ± 35	156 ± 93	NS
C_{max} (μg/mL)	64 ± 10	30 ± 8	S	28 ± 7	44 ± 8	S
t_{max} (min)	112 ± 39	94 ± 47	NS	84 ± 19	89 ± 28	NS
AUC_{0-t} (min × μg /mL)	(1.58 ± 0.32) x 10^4	(0.60 ± 0.23) x 10^4	S	(0.53 ± 0.09) x 10^4	(1.10 ± 0.23) x 10^4	S
$AUC_{0-\infty}$ (min × μg/mL)	(1.73 ± 0.7) x 10^4	(0.63 ± 0.24) x 10^4	S	(0.55 ± 0.12) x 10^4	(1.21 ± 0.51) x 10^4	S
Cl (μg /mL/min)	0.038 ± 0.007	0.041 ± 0.010	NS	0.050 ± 0.026	0.048 ± 0.018	NS
Bioavailability %	79.4 ± 4.7	40.0 ± 4.3	S	27.9 ± 4.9	55.6 ± 5.8	S

S: significant
NS: not significant

blood glucose level was significantly reduced (Fig. 2). Oral administration of MKC + gliclazide to diabetic rats treated with probiotics also resulted in a small but significant hypoglycemic effect (E_{max} = 9.34 ± 2.0) ($P < 0.01$). Interestingly, diabetic rats pre-treated with probiotics showed reduction in weight loss, urine production and water consumption, and improvement in behaviour (curious, active) and survival rate when compared with untreated diabetic rats.

Gliclazide pharmacokinetic parameters were not affected by the concomitant administration of MKC by the i.v. route in either healthy or diabetic rats with and without

probiotic pre-treatment (Fig. 3, Table 1). However, after oral administration, Cmax, AUC and F values of gliclazide were much lower in untreated diabetic rats than in untreated healthy rats. In contrast, Cmax, AUC and F values of gliclazide were much higher in probiotic treated diabetic rats than in probiotic treated healthy rats, and approached levels in untreated healthy rats (Fig. 4, Table 2). These values were similar with those of treated diabetic rats given gliclazide alone [38] suggesting that gliclazide systemic absorption in probiotic treated diabetic animals does not change as a result of the concomitant administration of MKC. Interestingly, C_{max}, AUC and F values of oral gliclazide in untreated healthy rats were higher when given with MKC. Moreover, in untreated healthy rats, the bioavailability of gliclazide increased from 54.4 ± 5.4 when given alone [9] to 79.4 ± 4.7 when given with MKC, while, in probiotic treated diabetic rats, gliclazide bioavailability was unchanged, 55.6 ± 5.8 (Table 2), *vs* 56.2 ± 6.4 [9], suggesting a different effect of MKC on gliclazide pharmacokinetics in diabetic animals.

4. Discussion

In previous in vivo studies, we have shown that MKC had a hypoglycemic effect when administered to alloxan-induced diabetic rats [8], and the combination of gliclazide and MKC had a synergistic hypoglycemic effect on diabetic rats which was greater than MKC alone [8,9]. Furthermore, in another in vivo study, we have shown that probiotic treatment had a hypoglycemic effect in alloxan-induced diabetic rats when given at the early stages of the disease [38]. Accordingly, in this in vivo study, we investigate the influence of probiotic treatment followed by the oral administration of gliclazide + MKC, on blood glucose levels while monitoring gliclazide pharmacokinetic parameters in healthy and diabetic rats.

At the start of experiments, all baseline (E0) values for untreated and probiotic treated healthy and diabetic rats were comparable. Intravenous administration of gliclazide + MKC to healthy and diabetic rats with and without probiotic pre-treatment produced little hypoglycemic effect. However, oral administration of gliclazide + MKC to diabetic rats produced a significant hypoglycemic effect suggesting the hypoglycemic effect of gliclazide + MKC in probiotic treated diabetic rats is due to the activation of MKC in the gut. The orally administered combination of gliclazide and MKC produced a greater hypoglycemic effect in diabetic rats than MKC alone. This synergistic effect of gliclazide after oral application could be due to gliclazide enhancing the production and/or the absorption of MKC active metabolites in the gut. The oral administration of MKC + gliclazide to probiotic treated diabetic rats also produced a significant reduction in blood glucose levels from 12.6 ± 2.0 mmol/L to 9.3 ± 2.0 mmol/L ($P < 0.01$). This may suggest probiotic treatment enhances the activation of MKC metabolism in a similar way to the reported bacterial activation of some primary bile acids [39,40]. On the other hand, in healthy rats treated with probiotics, neither gliclazide, MKC or MKC + gliclazide had an effect on blood glucose levels suggesting the presence of efficient compensatory mechanisms including glycogenolysis, glycogenesis, and gluconeogensis. Overall, pre-treatment with probiotics then subsequent oral administration of MKC + gliclazide to T1D rats resulted in optimum control over hyperglycemia as well as improved signs and symptoms in those diabetic animals.

Probiotic pre-treatment reduces the bioavailability of gliclazide after oral dose of gliclazide + MKC in healthy rats while the same probiotic pre-treatment increases the bioavailability in diabetic rats. The decrease in gliclazide bioavailability in healthy rats caused by probiotic treatment can be explained in the following way. Firstly, probiotics may activate efflux drug transporters that influence gliclazide absorption. Secondly, probiotics cause the formation of a 'thicker' layer of adherent mucous in the GIT [38,41], or improve the tightness of the intestinal barrier [42]. This newly formed 'bacterial' layer reduces the ability of gliclazide to reach the mucosal membrane of the enterocytes resulting in less drug being absorbed. Lastly, probiotics may bind free MKC

in a similar way to that reported between cholic acid and Lactobacilli [43] and consequently restricting gliclazide + MKC access to site of absorption in the gut. On the other hand, the increase in gliclazide bioavailability in diabetic rats caused by probiotic treatment can be explained by the following. Firstly, probiotic treatment can restore the activity of drug transporters responsible for MKC uptake. These drug transporters we have shown in previous ex vivo studies [18,44,45] to be impaired in tissues from alloxan-induced diabetic rats. Secondly, this increase in gliclazide bioavailability could also be the result of restoring the disturbed gut motility associated with diabetes [46] by probiotic treatment. Overall, the reason for the lower bioavailability of gliclazide in diabetic rats is unknown and awaits further research but one possible explanation is that changes in metabolic activation by the gut flora or in drug transporter activity and/or expression occur in diabetic animals.

In conclusion, T1D has been characterized mainly by glucose imbalance. Insulin has been the main stream treatment for T1D, but despite good control over blood glucose levels, T1D complications still occur. Accordingly, optimum control over blood glucose, maintaining tissues functionality and minimizing T1D complications can significantly optimize patient health status and well being. In recent years, probiotics and bile acids emerged as potential candidates that can be used as adjuvant agents to improve the treatment of diabetes.

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