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Evaluation of safety and tolerance of microencapsulated *Lactobacillus reuteri* NCIMB 30242 in a yogurt formulation: A randomized, placebo-controlled, double-blind study

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ABSTRACT

Probiotic organisms have shown promise in treating diseases. Previously, we have reported on the efficacy of microencapsulated *Lactobacillus reuteri* NCIMB 30242 in a yogurt formulation at lowering serum cholesterol levels in otherwise healthy hypercholesterolemic adults. This study investigates the safety and toxicology of oral ingestion of microencapsulated *L. reuteri* NCIMB 30242 in a yogurt formulation. A randomized group of 120 subjects received a dose of 5×10^{10} CFU microencapsulated *L. reuteri* NCIMB 30242 in yogurt ($n = 59$) or placebo yogurt ($n = 61$) twice/day for 6 weeks. Clinical chemistry and hematological parameters of safety were analyzed. Fecal samples were collected at these time points for the analysis of deconjugated bile acids. The frequency, duration and intensity of adverse events (AEs) and clinical significance of safety parameters were recorded for both groups. No clinically significant differences between the probiotic yogurt and placebo yogurt treated groups were detected in either the blood clinical chemistry or hematology results and there was no significant increase in fecal deconjugated bile acids ($P > 0.05$) between treated and control groups. The frequency and intensity of AEs was similar in the two groups. These results demonstrate the safe use of this formulation in food.

1. Introduction

The use of probiotic organisms dates back thousands of years with the use of traditional fermented milk products by indigenous peoples. Metchnikoff first recognized the health-promoting properties of these products, attributing their beneficial effects to the fermenting microbes contained therein thus introducing the concept of useful bacteria (Dobrogosz et al., 2010). Indeed, this concept has been supported innumerable times in the scientific literature demonstrating the efficacy of probiotic organisms to treat a variety of gastrointestinal, immunological and metabolic conditions in a number of models subjects including humans (Gareau et al., 2010; Narayan et al., 2010; Parkes et al., 2010; Sanz et al., 2010; Vanderhoof and Mitmesser, 2010). These studies highlight the potential probiotic organisms have to impact human health. To capitalize on their potential clinical applications however, more clinical evidence demonstrating not only the efficacy but the safety of these organisms is needed.

Probiotics are defined as live microorganisms that, when ingested in adequate amounts, are expected to confer health benefits to the host (WHO, 2001). In practice, species belonging to the lactic acid producing group of bacteria (LAB), such as *Lactobacillus*, *Bifidobacteria*, *Leuconostoc* and *Pediococcus*, are used most frequently and have a long history of use in fermented foods including yogurt, cheese, salami and olives (Salminen and von Wright, 1993; Kurmann et al., 1992). This long history of use in combination with their commensal status in humans and their environmental ubiquity suggests that these species are safe for consumption by

Abbreviations: AE, adverse events; ALT, alanine transaminase; APA, alginate-poly-L-lysine; AlkP, alkaline phosphatase; AST, aspartate aminotransferase; Bpm, beats per minute; BSH, bile salt hydrolase; BMI, body mass index; Ca^{2+}, calcium; Cl^-, chloride; CSV, clinically significant value; GGT, gamma-glutamyl transpeptidase; ICH–GCP, International Conference on Harmonization – Good Clinical Practice; LAB, lactic acid bacteria; N, number of subjects; ANOVA, one-way analysis of variance; PRO, patient-reported outcome; PO_4^{3-}, phosphate; K^+, Potassium; QC, quality control; QPS, qualified presumption of safety; Na^+, sodium; SOP, standard operating procedures.

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humans. *Lactobacillus reuteri* was first isolated in the early 1960s from the human gastrointestinal tract; since then, additional species have been isolated from the intestinal tracts of healthy non-human species as well as in traditional food sources (Lee et al., 2009; Molin et al., 1992; Naito et al., 1995; Taranto et al., 2003). A large number of human clinical studies have shown no adverse effects associated with the consumption of *L. reuteri* (Wolf et al., 1998; Oliva et al., 2011; Wolf et al., 1995; Weizman and Alsheikh, 2006; Rosander et al., 2008; Coccorullo et al., 2010; Connolly et al., 2005). Accordingly, *L. reuteri* has a qualified presumption of safety (QPS) with the European Food Safety Authority (EFSA Panel on Biological Hazards, 2009).

To date, the use of many probiotic formulations has focused on the non-specific benefits derived from the action of the probiotic organism on maintaining a favorable balance of gastrointestinal flora (Parkes et al., 2010; Bosscher et al., 2009; Vasile et al., 2011; Rauch and Lynch, 2011). However, there is increasing interest in developing more efficacious strains of probiotics tailored to treat specific conditions. In this regard, we have selected *L. reuteri* NCIMB 30242 for its cholesterol lowering capabilities. This organism possesses a highly active bile salt hydrolase (BSH) enzyme enabling it to efficiently deconjugate bile acids (Jones et al., 2011). The safety features of *L. reuteri* NCIMB 30242 have been extensively characterized at the molecular, metabolic and genomic levels (Branton et al., 2011). The genome of *L. reuteri* NCIMB 30242 does not encode virulence factors that may implicate it in any opportunistic infections, nor antibiotic resistance determinants which can be transferred to other bacteria (Branton et al., 2011). The strain does not produce metabolic by-products such as biogenic amines and D-lactate at levels that can produce adverse effects in sub-sets of the population (Branton et al., 2011). Based on these intrinsic features of *L. reuteri* NCIMB 30242, its consumption was not expected to produce harmful effects in humans.

In addition to efficacy and safety, the delivery of probiotic organisms is an important consideration. Exposure to acidic compounds, oxygen, digestive enzymes and other antibacterial components can compromise the survival of probiotics as they transit the gastrointestinal tract (Holzapfel et al., 1998; Huang and Adams, 2004). Encapsulation of bacteria in alginate-poly-L-lysine (APA) microcapsules provides a physical barrier against immunoglobulins and digestive enzymes, buffers against an acidic gastric environment, and concentrates the bacteria within the microcapsule, resulting in the delivery of high numbers of metabolically active cells to the proximal small intestine (Del et al., 2011; Prakash et al., 2011).

The aim of the present study was to evaluate the safety and tolerability of APA microencapsulated *L. reuteri* NCIMB 30242 in a yogurt-based formulation in a randomized, double-blind, placebo-controlled study of closely-monitored human subjects. Included was sequential documentation of the clinical chemistry, hematology and fecal parameters of the treated subjects to determine whether the values obtained extended beyond normal limits or differed significantly from the findings in the placebo group. Additionally, the number, duration and intensity of AEs reported were closely monitored and the clinical significance of safety parameters was assessed.

2. Materials and methods

2.1. Study design

The objective of the study was to assess the tolerance, safety and impact on health parameters of mildly hypercholesterolemic human subjects to microencapsulated *L. reuteri* NCIMB 30242 in a yogurt formulation. The double-blind, placebo-controlled, multi-center study included 120 male and female subjects randomized into two treatment groups (probiotic yogurt or placebo yogurt). The subjects were screened to determine whether they met the inclusion criteria prior to a 2-week wash-out period in which subjects were advised to follow general dietary recommendations (Canada's Food Guide, Health Canada) throughout the entire study, a 2-week run-in period in which subjects consumed placebo yogurts twice daily with the morning and evening meals, and a 6-week treatment period in which subjects consumed either placebo or probiotic yogurts twice daily with the morning and evening meals. A variety of biological samples including blood and feces were taken at various time points from the participants for analyzes. The study was performed by a Contract Research Organization, A-Pharma s.r.o. (Prague, CZ) in compliance with International Conference on Harmonization – Good Clinical Practice (ICH-GCP), including the archiving of essential documents. The study was conducted according to the principles in the Declaration of Helsinki. All procedures involving human subjects were approved by the central ethics committee (multi-centric ethics committee) and the local ethics committee in the Czech Republic. Written informed consent was obtained from all subjects. Regular monitoring visits to the investigational sites were made prior to, during and post-treatment phase by A-Pharma s.r.o. monitors to insure that the study was conducted in compliance with the protocol, the ICH-GCP guidelines and the applicable regulations for the clinical trial and that the rights, safety and well-being of the study subjects were protected at all times. The study was registered on ClinicalTrials.gov (NCT ID: NCT0118579).

2.2. Subject inclusion

Strict study entry criteria were assessed prior to enrollment into the study. Otherwise healthy hypercholesterolemic adult men and women were recruited from 5 centers in Prague, Czech Republic. Inclusion criteria for randomization were otherwise healthy males and females between the ages of 18 and 74 years old (inclusive); LDL-cholesterol levels >3.4 mmol/l; triglyceride levels <4.0 mmol/l; body mass index (BMI) of 22–32 kg/m^2. Exclusion criteria for randomization were the use of cholesterol lowering prescription drugs within the last 6 months; use of cholesterol lowering supplements within the last 3 months; history of chronic use of alcohol (>2 drinks/d); use of systemic antibodies, corticosteroids, androgens, or phenytoin; myocardial infarction, coronary artery bypass, or major surgical procedure within the last 6 months; lactose intolerance or allergies to dairy products; history of angina, congestive heart failure, inflammatory bowel disease, pancreatitis, diabetes, gastrointestinal, renal, pulmonary, hepatic or biliary disease, or cancer (evidence of active lesions, chemotherapy or surgery in the past year); chronic use of probiotics or fiber laxative (>2 doses/week), or stimulant laxatives; history of eating disorders; exercise greater than 15 miles/week or 4000 kcal/week; pregnancy, breast feeding, or intent to get pregnant.

2.3. Examination of physical condition and vital signs

Subject evaluations were performed at 5 different time points: Visit V0 (week -4), V1 (week -2), V2 (week 0, randomization and treatment baseline), V3 (week 3, treatment midpoint) and V4 (week -6, treatment endpoint). The documented demographic characteristics of the participants included medical and surgical histories, alcohol and tobacco use and methods of contraception (for females of childbearing age).Abbreviated physical examinations were conducted at visits V1 (week -2) and V4 (week 6). Parameters recorded included the subjects' weight, body mass index (BMI), vital signs (heart rate, systolic and diastolic blood pressure, and oral temperature) as well as any detectable abnormalities of the skin, eyes, ears, nose and throat, heart, lungs, abdomen, musculoskeletal system, lymph nodes and nervous system. The subjects' dietary compliance and use of alcohol was also regularly assessed throughout the study period.

2.4. Preparation of test material

L. reuteri NCIMB 30242 (Cardioviva™) was propagated in a FV8 fermenter and concentrated in compliance with standard operating procedures (SOP) and quality control (QC) procedures at Microbial Developments Ltd. (Malvern, UK). Microbiological analyzes and bacterial culture purity were confirmed after each production batch. Alginate-poly-L-lysine-alginate (APA) microcapsules containing *L. reuteri* NCIMB 30242 were prepared in compliance with SOP and QC procedures at Brace GmbH (Karlstein, DE) to a viability of 5×10^9 CFU/g microcapsule which was confirmed after each production batch. Placebo and treatment yogurts were produced and prepared at Milcom (Prague, CZ). Placebo yogurts contained: 7.9 g protein; 11.5 g carbohydrates; 1.3 g lipids; and 1.25×10^9 yogurt bacteria. Probiotic yogurts contained: 7.2 g protein; 10.6 g carbohydrates; 1.2 g lipids; and 1.15×10^9 yogurt bacteria; and 10 g microencapsulated *L. reuteri* NCIMB 30242. In addition to bacterial loading, *L. reuteri* NCIMB 30242 APA microcapsules contained ~0.03 g sodium alginate/g microcapsules, 0.0005 g poly-L-lysine/g microcapsules, calcium chloride (trace) and water. The viability of *L. reuteri* NCIMB 30242 in probiotic yogurts was determined at the batch expiry date (after international shipping at 4 °C) and was $\sim 1.4 \times 10^9$ CFU/yogurt. Based on production and expiry viability values, and previous stability studies, the average viable delivered dose of *L. reuteri* NCIMB 30242 was estimated to be $\sim 1 \times 10^{10}$ CFU/yogurt. Placebo and probiotic yogurts were produced 5 times during the study with the batch numbers 1 through 5. The expiry date of placebo and probiotic yogurts was maintained at 3 weeks after yogurt production.