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SAFETY AND PROTECTIVE EFFECT OF *BIFIDOBACTÉRIUM SPP. USED* AS PROBIOTIC AGENT *IN VIVO* AGAINST ENTEROPATHOGENIC *ESCHERICHIA COLI*

Fatima MAHMOUDI*, Miloud HADADJI, Kheira BENLAHCEN

Department of Biology, Faculty of Sciences, Laboratory of Applied microbiology, University of Es-Senia of Oran, Box 16, Es-Senia 31100, Oran, Algeria

*Corresponding author: mahmoufati@yahoo.fr

Abstract: This study showed the effect of fermented milk with *Bifidobacterium bifidum* on the contaminations by *Escherichia coli* enteropathogenic EPEC and intestinal disorders. The *B. bifidum* strain (Bf) was isolated on MRS medium containing 0.5g/L of cysteine hydrochloride and 2 mg/L of nalidixic acid. This strain was isolated from breast-fed infant feces. This strain is considered as a model strain, for antagonism *in vivo* developed in the rat (wistar). It has has been demonstrated that the administration of *Bifidobacterium* strain cause a significant reduction of the rate of *E. coli* enteropathogenic multiplication in the rats feces. Results of the macroscopic study of histological sections have confirmed the positive effect of treatment with bifidobacteria. In this study, the effect of *Bifidobacterium* addition in reducing the enteropathogenic bacteria effect in the digestive tract was confirmed by in vitro and in vivo conditions.

Keywords: *Bifidobacterium*, inhibition of enteric pathogens, antagonism effect.

Introduction

The human intestinal microbiota constitutes a complex ecosystem, which is now well recognized for its impact on human health and well-being (Doré *et al.*, 2010). More than 400 species within the intestinal microflora can be identified and may attain population levels nearly as high as 10^{12} g/L in the colon (Mitsuoka, 1989). Bifid microflora has been particularly studied for its ability to protect against diarrhea and various diseases. Indeed, bifidobacteria have an antagonistic effect against pathogens. Bifidobacteria are bacilli Gram positive belonging to the dominant gut microbiota in humans and animals. In recent years, Bifidobacteria have attracted considerable attention due to their overall beneficial effects on health (Peter *et al.*, 2001); they play a significant role in maintaining the balance of intestinal microflora by correcting intestinal disorders. However, the composition of this flora may be altered by various factors in food and environmental, that makes the host organism susceptible to disease or digestive disorders. The composition of the dominant species of the indigenous bifidobacteria varies by age. *B. lactis, B. longum, B. breve* and *B. parvolorum* are found during childhood. They are replaced in the adulthood by *B. adolescentis, B. catenulatum, B. pseudocatenulatum* and *B. longum* (Ariane *et al.*, 2010).

Infection with enteric pathogens continues to be a health problem worldwide, especially in children. Intestinal epithelium provides the first line of defense of the organism, providing an efficient barrier against pathogens and macromolecules. The

mucus layer and the resident gut microbiota protect the gut mucosa from adhesion and invasion of pathogens (Candela *et al.*, 2010). Therefore, probiotics have been proposed for prevention and treatment of gastrointestinal tract (GIT) infections (Rodrıguez *et al.*, 2012).

The work of Metchnikoff (1907) has shown that consumption of fermented foods can restore the intestinal flora by generating beneficial effects on human health and animals. Recent investigations have highlighted the crucial role of intestinal microflora in maintaining and improving health. Some of these studies have shown that animals with a conventional complete intestinal microflora are more resistant to infection than animals axenic (germ-free).

Bifidobacteria have gained a lot of attention because of their association with numerous health-promoting effects, even though some mechanisms of these beneficial effects remain unexplained (Turroni *et al.*, 2009). Thus, various bifidobacterial strains are currently used as probiotics in functional food products, and selecting new probiotic strains is of great interest (FAO/WHO, 2002). These strains must display several characteristics, one of which is that they must be of human origin. Therefore, in the perspective of either understanding the mechanisms of the beneficial effects of bifidobacteria or strain selection for probiotic uses, reliable enumeration and isolation of bifidobacteria from human feces are needed (Ferraris *et al.*, 2010). These bacteria colonize the neonatal intestine from the first week after birth and inhabit the gastrointestinal tract throughout life, where they contribute to human health and well-being (Turroni *et al.*, 2009). This study is intended to observe the intestinal disorder and treatment of diarrhea by probiotics (*B. bifidum*).

Materials and methods

Bacteria strains

The *B. bifidum* strain (Bf) was isolated on MRS medium containing 0.5g/l of cysteine hydrochloride and 2.0 mg/L of nalidixic acid. This strain was isolated from breast-fed infant faeces, aged less than five months and then was incubated an anaerobiosis for 48 h at 37°C. This species was selected because of its antagonistic effect on EPEC (Gibson and Wang 1994), its resistance to gastric juice (Biavati *et al.*, 1992) and its capacity to survive at high rates in the intestine (Marteau *et al.*, 1992). *E. coli* EPEC strain was isolated from infant diarrhea and then incubated in EMB (Eosin and Methylene blue, IPA) medium in aerobiosis conditions for 48 at temperature of 44°C.

Culture conditions and growth

Culture conditions

MRS Agar containing 0.5 g/L of cysteine Hcl (Merck) and 0.2 g/L of nalidixic acid was used for microbiological analysis of *B. bifidum*. EMB base medium was used for isolation of EPEC rat's feces. In order to count the number of living EPEC in feces (in days) the colonies were violet colored with a metallic luster on EMB medium.

Rats

The experiments were carried out on females of the same species (Wistar strain) and of the same age (30 days), weighing between 150 and 160 g at the beginning of the experiment. Rats were kept separately in metal cages (50 cm) in a well-aired animal house at a constant temperature of 21 ± 1°C, and 12 h of light. The rats were fed on basal diet with ad libitum.

Preparation of infant milk and the fermented milk

The milk used in this study was Gigoz infant milk. A quantity of 60 g of milk powder was dissolved in 400 mL of distilled water. The milk fermented with *B. bifidum* and with EPEC inoculum was prepared every day.

B. bifidum ferment was prepared as follows: 2 colonies were inoculated in 9 mL of the prepared infant milk and incubated for 18 h at temperature of 37°C. Another 2 colonies of *E.coli* were inoculated in 9 mL of prepared infant milk and incubated for 18 h at 44°C.

Analysis of rats intestinal flora

A number of 30 rats were separated into 4 batches of 6 rats, with each batch received a treatment (standard inoculated germ, duration of treatment).

Before starting the study, a search was made for EPEC and *B. bifidum* in the fecal flora of rats and the number of *Enterobacteria* was counted.

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Collection of feces

Feces were recovered at each 4 h after treatment every day during the period of the fermented milk ingestion by the young rats (1 g of stools was diluted in 9 mL of physiological water (9 g NaCl/mL) and 1 mL of the suspension was used for analysis.

Rats inoculation with the tested strains

Control group: Samples collected before administration of *B. bifidum* (Bf) and *E. coli*.

Batch 1: The rats received 1 mL of milk inoculated with 10^8/mL *E. coli* for one week.

Batch 2: The rats received 1 mL of milk inoculated with 10^8UFC/mL of *B. bifidum* for the first week and 1 mL of milk inoculated with 10^8UFC/ mL of *E. coli* during the second week.

Batch 3: The rats received 1 mL of milk inoculated with 10^8UFC/mL of *E. coli* for 48 h, then, they received 1ml of fermented milk with 10^9UFC/mL of *B. bifidum* for one week (Doumandji, 2007).

Microbiological analysis

Colonies of *B. bifidum* and *E.coli* stains were counted daily during and after treatments for a period of two weeks. The purpose of these analyses was to determine the number and survival of *B. bifidum* and EPEC strains. Decimal dilutions were performed. A volume of 1 mL of dilution was added in the MRScyst medium for the *B. bifidum* strain (incubated for 48 h at temperature of 37°C under anaerobiotic conditions). Another volume of 1 mL of dilution was distributed on the surface of EMB plates for the EPEC strain (incubated at 44°C under aerobiotic conditions).

Results and discussion

Studies in vitro

Bifidobacteria were detected using MRScys medium (Mahmoudi *et al.*, 2013). The selectivity is due to the presence of (0.1 g) mupirocin, bifidobacteria being antibiotic resistant. Acetic acid is the second selective agent for anaerobic Gram-positive bacteria. This medium has been used for the isolation and counting of *Bifidobacterium* in rats and poultry (Rada Sirotek *et al.*, 1999).

In Figure 1, the evolution of the *E. coli* enteropathogenic rate in the three studied batches is represented.

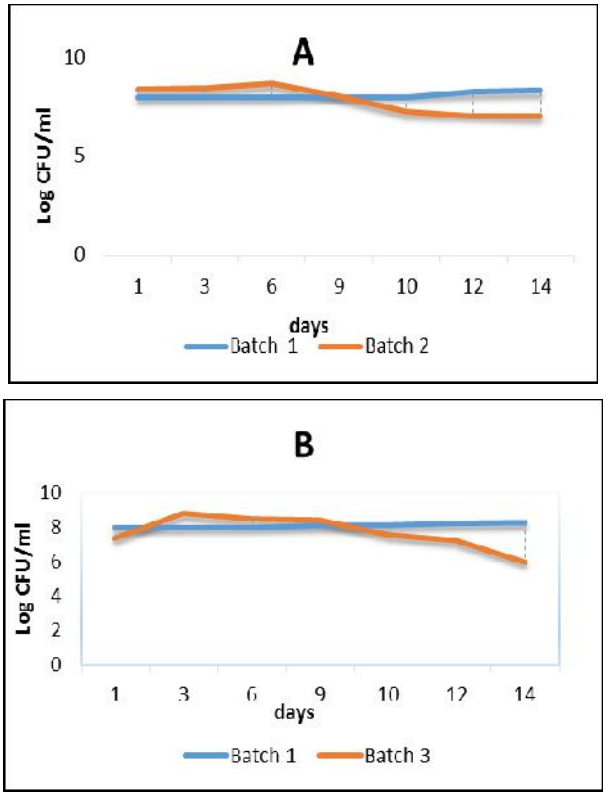

Figure 1. *Changes in the number of EPEC in rat feces in the different batches (A and B)*

As can be seen, after preventive treatment with B. *bifidum* (Figure 1A), the number of *E. coli* in the feces decreased after the 8th day in rats. After 14 days of treatment a number of 10^7 UFC/mL *E. coli* was present. After the therapeutic treatment of batch 3 (Figure 1B), the number of EPEC increase from 10^7 UFC/ml to 10^9 UFC/ml on the 4th day and then the number decreased to 10^6 UFC/mL.

However, *B. bifidum* was able to survive in the intestine and to exert a probiotic effect throughout the period of ingestion and even for a few days after ingestion ended. In this situation, we can anticipate their persistence after stopping the protocol according to Neish, (2002) that have shown the efficiency of taking into account bifidobacteria in the treatment of infectious diarrhea, notably pseudo membranous colitis.

Macroscopic study

Observations of small intestine after dissection of the rats were done as follows: control rats (no treatment), the samples were of uniform size (0.3

cm). It was observed that the intestinal lining was in good condition during dissection.

Batch 1: Small intestines presented dark brown colour, accompanied by poor odour during the dissection with a contraction of the organs (in particular intestines) with 0.2 cm in diameter, compared to those observed in control rats (0.52 cm in diameter) (Figure 2).

Batch 2: The small intestines have the same characteristics (no change in color, appearance, diameter and volume) as those observed in the control rats (0.4 cm diameter).

Batch 3: The appearance (shape and color) and contraction were smaller than those observed for rats (batch 2 and 2nd dissection).

The results of the macroscopic study showed that control batch of rats presented severe infection accompanied by a marked contraction of intestinal mucosa (intestinal atrophy), probably due to contamination by EPEC. These symptoms were not present for batch 3, and after administration of bifidobacteria (2nd dissection) (Figure 2).

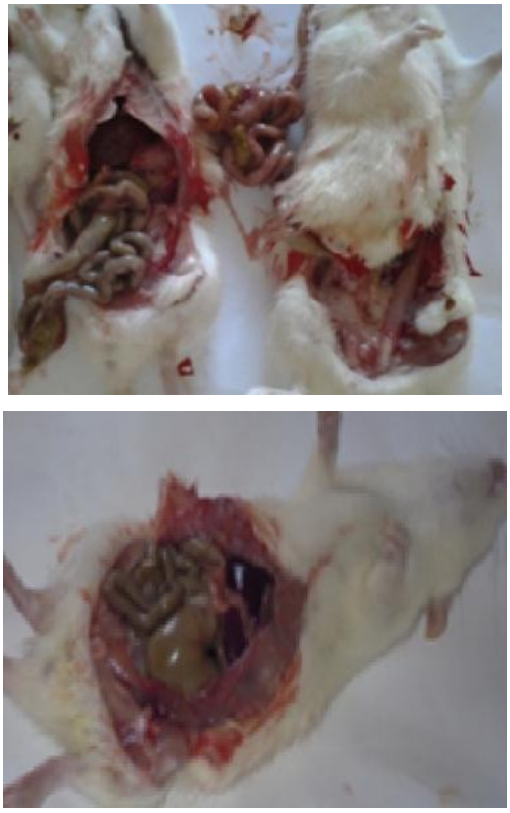

Figure 2. *Macroscopic observations of the small intestine of rats (batches 1, 2 and 3) after dissections*

Conclusions

The significant increase in the number of *Bifidobacterium* suggested that the strain has resisted the gastro-intestinal passage. Our results showed that the survival of *B. bifidum* in the digestive tract until the fifth day after ingestion was sufficient for *B. bifidum* to exercise its probiotic effect.

In this work, we also records that the administration of bifidobacteria strains causes a significant decrease in the rate of *E.coli* enteropathogenes in rats feces. The clinical study conducted on human subjects (aged 6-48 years) showed positive effect of *Bifidobacteria* stains administration on the clinical symptoms of irritable bowel syndrome (Mastrandrea *et al*. (2004).

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