# *Lactococcus lactis* NCDO2118 produces anti-hypertensive GABA and exerts acute hypotensive in spontaneously hypertensive rats

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**Abstract**

Gamma (γ)-aminobutyric acid (GABA) is a molecule with nutraceutical interest since it is recognized for its health-promoting properties such as hypotensive effects. Some lactic acid bacteria (LAB) have been shown to be able to produce GABA. In the present investigation, *Lactococcus* (*L.*) *lactis* NCDO2118, a probiotic LAB, was cultured in a medium supplemented with different concentration of glutamate, the substrate for GABA production. This strain was evaluated in SHRSP (Spontaneously Hypertensive Stroke-Prone Rats) to assess its hypotensive effect. The evaluation showed that GABA production is variable depending on the glutamate concentration and incubation time and that the increased GABA production potentiates its hypotensive effect. This work demonstrates that *L. lactis* NCDO 2118 could be used as a tool for developing health-promoting foods, such as those enriched with GABA as an interesting and novel pharmacological strategy.

**Keywords**

*Lactococcus lactis*; Gamma-AminoButyric Acid; GABA; Glutamic Acid Decarboxylase; GAD; Hypertension

 

## 1. Introduction

Cardiovascular diseases are responsible for approximately 17 million deaths per year, and more than half of the cases are due to complications of hypertension. High blood pressure, can lead to the damage of the kidneys, eyes, arteries, heart, and brain, thereby affecting cognitive ability and vital biological functions. Changes in diet and lifestyle, as well as adherence to medication, may help in a better control of hypertension (World Health Organization, 2013).

Gamma-aminobutyric acid (GABA) is a non-protein four-carbon amino acid responsible for many beneficial properties on human health, including anti-hypertensive effects. When GABA was injected in the lateral ventricle in an animal model, the arterial pressure and heart rate were decreased (Sasaki et al., 1986). The oral route is also a promising strategy for GABA administration, both in subclinical as well as clinical trials (Elliott & Hobbiger, 1959; Inoue et al., 2003; Takahashi, Tiba, Iino, & Takayasu, 1955).

Lactic acid bacteria (LAB) are the principal microorganisms that produce GABA (Li & Cao, 2010). This capability is due to the performance of glutamate decarboxylase (GAD, EC 4.1.1.15), an enzyme localized in the cytoplasm that is recruited to the membrane when the pH falls. In the cellular membrane, GAD performs the irreversible alpha-decarboxylation of L-glutamate resulting in GABA production. In this context, GABA can increase ATP production and acid tolerance (Higuchi, Hayashi, & Abe, 1997).

Among the factors that affect the production of GABA by bacteria are temperature, pH, incubation time and different media additives (Gomaa, 2015). The aim of this work was to evaluate different conditions of growth for the bacteria *Lactcococcus lactis* NCDO2118, a known GABA-producing LAB (Mazzoli et al., 2010), on GABA production and

 

evaluate its anti-hypertensive potential. For this latter objective, the bacteria were orally administered in an acute hypertension pratical model using SHRSP (Spontaneously Hypertensive Stroke-Prone Rats).

## 2. Material and Methods

### *2.1. Bacteria and growth conditions.*

*L. lactis* subsp. *lactis* NCDO 2118 (NCDO) was used in this study. It was activated at 30°C in M17 medium (Difco), containing 0.5% glucose (w/v) (GM17) for 24 hours. Afterwards, bacteria were inoculated 1:10,000 (v/v) in GM17, supplemented or not with glutamate (0.5% and 5% (w/v)) and incubated for 48 hours at 30°C. These cultures were prepared freshly every day during the test period.

### *2.2. Lactococcus lactis in glutamate culture.*

To evaluate the effects of glutamate on *L. lactis*, growth, optical density, pH, colony-forming unit (CFU/mL) and GABA production were determined at four time points: 0, 6, 24 and 48 hours' incubation at 30°C. Optical density was measured at 580 nm ($OD_{580}$), the pH in digital pH meter, and serial dilutions were performed in GM17 medium and plated in triplicate in solid medium containing 1.5% (w/v) agar to determine CFU/mL after incubation at 30°C for 48 hours. GABA-concentrations in the culture supernatants were determined using the enzymatic GABAase assay as described previously using a commercial kit (Tsukatani, Higuchi, & Matsumoto, 2005).

### *2.3. Animals*

Male SHRSP rats (Masineni, Chander, Singh, Powers, & Stierjr, 2005) 20-24 weeks old (weighing between 245 and 320g) were obtained from Animal House of Hypertension Laboratory of Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais (UFMG). All animals received a balanced

 

diet *ad libitum*. The experimental groups consisted of a minimum of 5 rats, and the protocols were accepted by the animal protection committee and Ethics Center on Animal Use of UFMG.

### *2.4. Femoral canulation of spontaneously hypertensive rats*

The procedure for catheterization was performed as previously described (Meyer & Fish, 2005). Briefly, the animals were anesthetized intraperitoneally with 2.5% of tribromoethanol, a small incision was performed in the inguinal region of the isolated femoral artery and a polyethylene cannula of 15 cm PE50 to 4 cm PE10 was made and implanted into the abdominal aorta through the femoral artery. After the catheter was secured and exteriorized subcutaneously into the dorse of the animal in the cervical region and the incision was sutured, physiological solution (0.9% NaCl, (w/v)) plus heparin (100 U.I/0.1 mL) was administered. The cardiovascular parameters of the animals were monitored after 24 hours of surgery.

### *2.5. Oral treatment and monitoring of cardiovascular parameters in Biopac System*

Pulsatile arterial pressure (PAP) was recorded by a signal sent to a transducer connected to the cannula inserted into the abdominal aorta. The pressure fluctuations were amplified and converted into signals captured by a data acquisition system (Biopac System, model MP150) and using the software Acqknowledge v.3.5.7 (Biopac System). Mean arterial pressure (MAP) and heart rate (HR) were recorded every 10 minutes during 6 hours. After the first hour of stabilization of cardiovascular parameters, the animals received 1 mL of single oral administration of LAB by gavage. The monitoring was performed until the sixth hour after treatment administration (Figure 1).

 

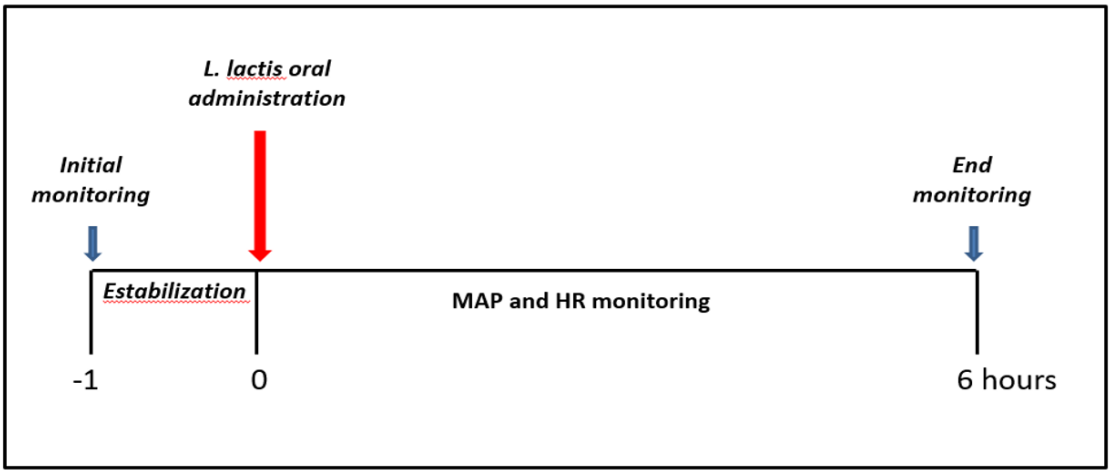


**Figure 1.** Experimental protocol.

### *2.6. Monitoring of Blood Pressure by Radiotelemetry*

The animals were anesthetized with tribromoethanol 2.5% (2.5 mg/kg), the catheter-transducer was fixed into the abdominal aorta just above the bifurcation of the iliac arteries, and the sensor was fixed to the abdominal wall. The animals were used in the experiment after 10 days. Arterial pressure was monitored by radio frequency for 30 hours using a telemetry system (Data Sciences International, MN; model TA11-PA C40). The software (Dataquest A.R.T., Gold 2.0) acquired the data to be analyzed.

### *2.7. Statistical Analysis*

Data were analyzed using two-Way ANOVA followed by a Bonferroni posthoc test, and $P<0.05$ was considered significant. Calculations were performed using the GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA).

## 3. Results

### 3.1. *Effect glutamate in L. lactis culture*

*Lactococcus lactis* exhibits a different behavior when cultured in GM17 medium supplemented with 0.5% or 5.0 % of glutamate. The optical density, pH and

 

GABA concentration of supernatant were monitored during 48 hours (Figure 2). The optical density presented larger increase in the 6 hour slot in both culture conditions, while in 24 hours the microorganism were already in the stationary growth phase. No growth was observed in the control containing only the culture medium. The lowest pH values were observed after the 24 hour and a small increase (of 3 and 7%) was observed in the 48 hour of cultures NCDO in GM17 containing 0.5 and 5.0% glutamate respectively (Figure 2).

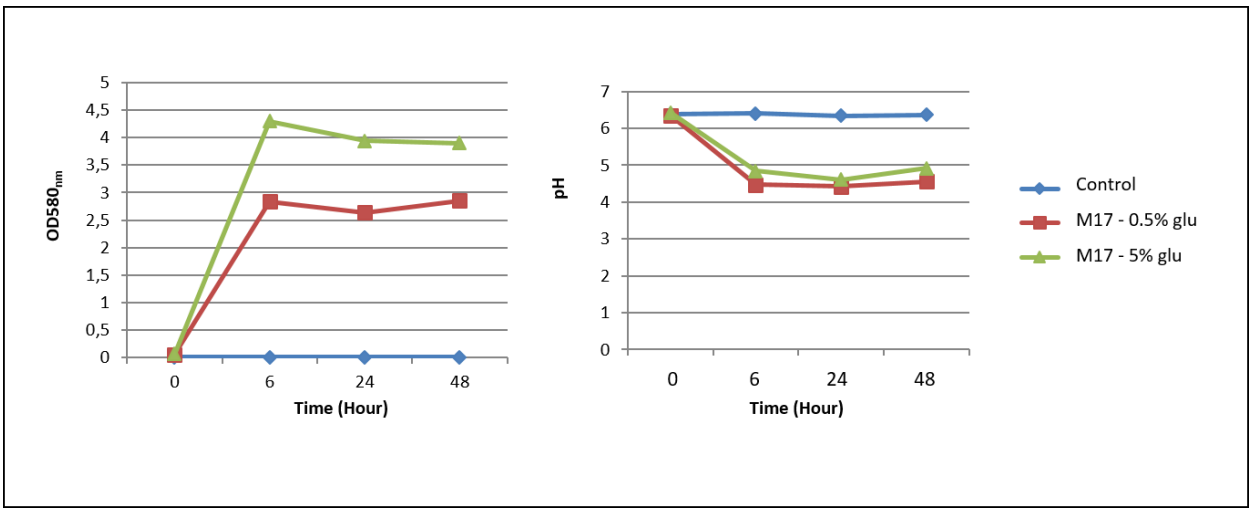


**Figure 2.** Glutamate effect in *Lactococcus* culture.

The production of GABA showed a progressive increase over time. After 24 hours incubation, 30 or 28 mM GABA was produced when *L. lactis* was grown with 0.5 or 5.0 % glutamate respectively. The highest yield was observed after 48 hours incubation, there was a higher yield with 48 and 65 mM, respectively (Figure 3).

 

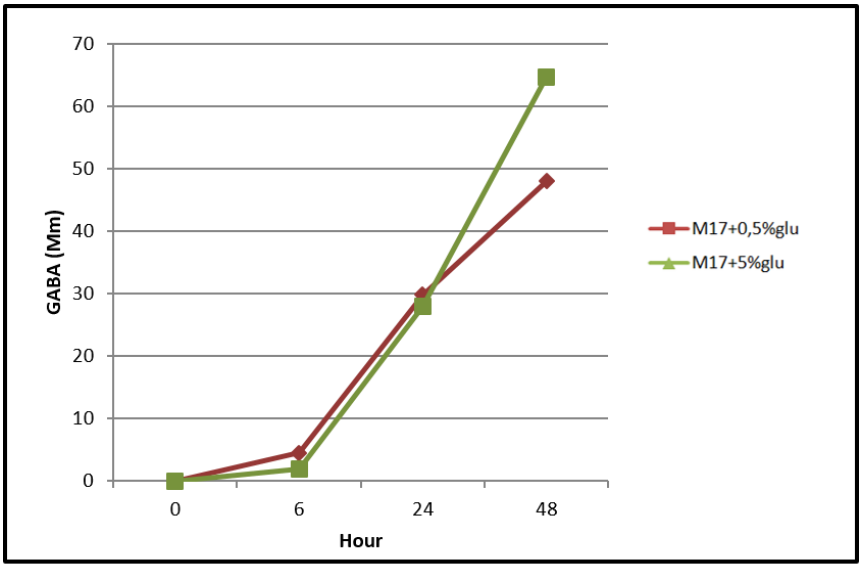


**Figure 3.** GABA *Lactococcus lactis* NCDO 2118 production.

### 3.2 *Potential hypotensive effect of GABA-producing Lactococcus lactis*

To evaluate the hypotensor potential effect of *L. lactis* NCDO2118, the bacterial culture was supplemented or not with 5% glutamate. After 48 hours of growth, 1 mL of *L. lactis* was administered orally in SHRSP animals. The arterial pressure was monitored continuously for 6 hours at 10 minutes' intervals. Figure 4 presents the results comparing *L. lactis* groups with (NCDO GGM17) and without (NCDO GM17) glutamate supplementation. The hypertensive animals treated with only the strain (grown without glutamate) showed a gradual decrease of arterial pressure starting at 60 minutes up to 200 minutes post-administration, where a 22 mmHg decrease was observed. Past this point, a gradual increase of blood pressure was observed until it returned to the initial level. The group with increased production of GABA (NCDO – GGM17), presented a progressive reduction in arterial pressure from the 40th minute with values that were significantly lower that those of the non-glutamate induced group. In this group, the arterial pressure was reduced up

 

to 28 mmHg after 300 minutes post-inoculation with the microorganisms, and this blood pressure remained constantly lower during the entire evaluation time. Comparing the effect produced by the two groups on the reduction of hypertension, a statistical difference was found between treatments.

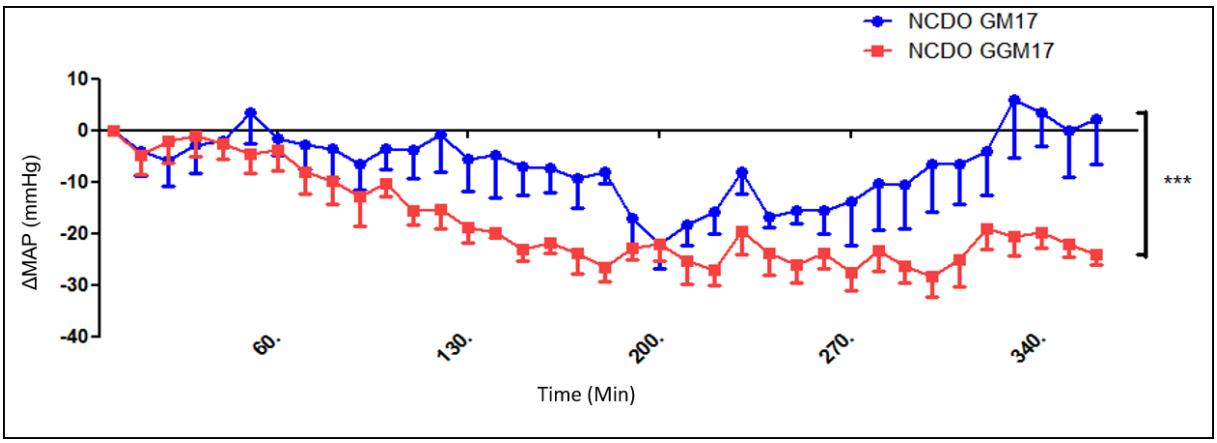


**Figure 4.** Graphical depiction of Mean Arterial Pressure (MAP) with *Lactococcus lactis* NCDO2218 treatment in SHRSP rats. NCDO GM17- hypertensive group, treated with *L. lactis* without glutamate supplementation; and NCDO GGM17- hypertensive group treated with *L. lactis* with glutamate supplementation. *** P<0.01

When administering either the supernatant or cell fractions in hypertensive animals, none of these fractions alone was able to produce the same effect that was observed when the whole culture of *L. lactis* grown with 5% glutamate was used. The treatment with entire culture was statistically more effective against hypertension than any of the isolated fractions (Figure 5).

 

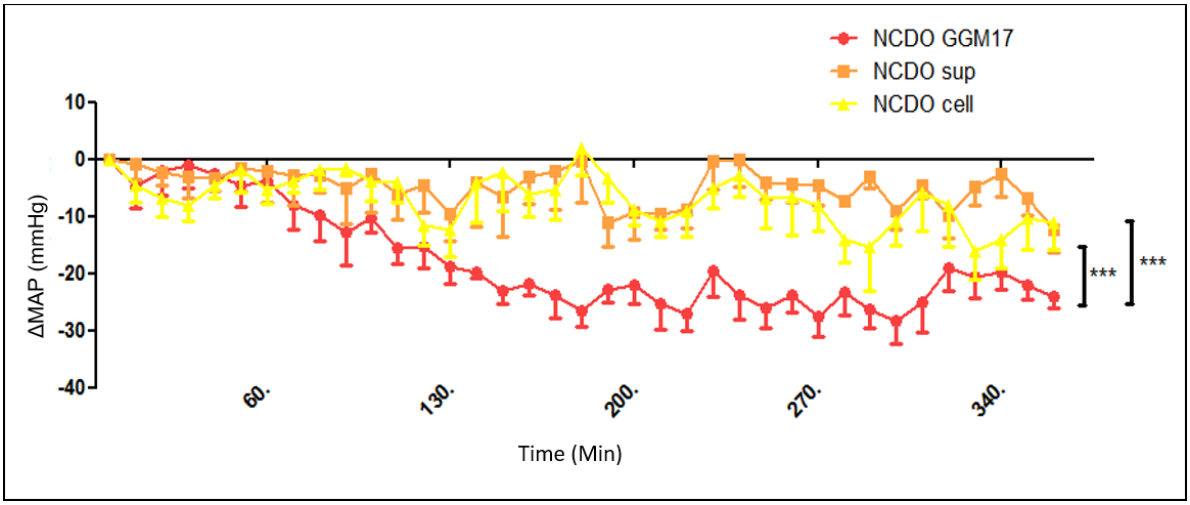


**Figure 5.** Graphical depiction of Mean Arterial Pressure (MAP) with *Lactococcus lactis* NCDO2218 culture and its fractions in SHRSP rats treatment. NCDO GGM17- hypertensive group, treated with *L. lactis* with glutamate supplementation; NCDO sup - hypertensive group treated with supernatant of *L. lactis* culture with glutamate supplementation; and NCDO cell - hypertensive group treated with cell fraction of *L. lactis* culture with glutamate supplementation. *** P<0.01

### 3.3. *Monitoring of blood pressure reduction by telemetry*

To evaluate the effect of *L. lactis* strain on elevated blood pressure in rats for more than 6 hours, a telemetry system was used and was monitored for 30 hours. During this period, the animals of all groups showed an increase in blood pressure from the tenth hour followed by a slight reduction (Figure 6).

 

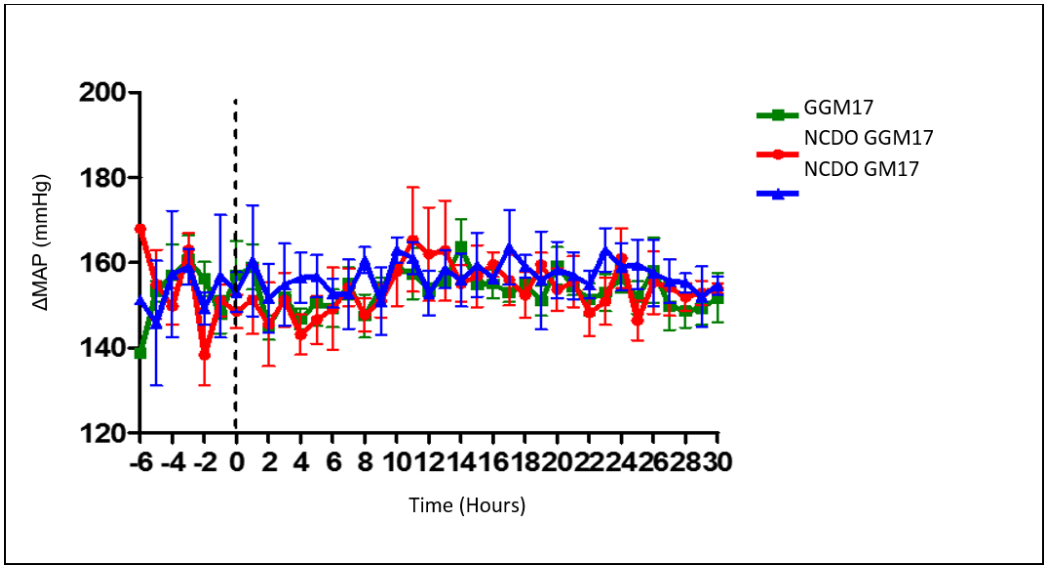


**Figure 6.** Graphical depiction of telemetry measurements to effect of the treatment with *Lactococcus lactis* NCDO2218 for 30 hours. GGM17 - hypertensive group treated with culture medium with glutamate supplementation; NCDO GM17- hypertensive group treated with *L. lactis* without glutamate supplementation; and NCDO GGM17- hypertensive group treated with *L. lactis* with glutamate supplementation.

## 4. Discussion

Gamma-Aminobutyric acid (GABA) is an amino acid that acts as an inhibitory neurotransmitter in the nervous system. The GABA system regulates important psychological processes, including emotional behavior (Bravo et al., 2011); dysfunction of such processes may involve anxiety and depression (Fatemi, Folsom, Rooney, & Thuras, 2013). GABA also regulates, among other functions, cardiovascular conditions such as blood pressure and heart rate (Cryan & Kaupmann, 2005; Mody, De Koninck, Otis, & Soltesz, 1994; Roberts & Frankel, 1950; Schousboe & Waagepetersen, 2007).

 

The ability of GABA in lowering blood pressure has been reported in animal and human trials. Its potential is attributed in part to blocking the peripheral ganglia, or possibly through the inhibition of noradrenaline release from sympathetic nerve endings (Elliott & Hobbiger, 1959; Hayakawa, Kimura, & Kamata, 2002; Stanton, 1963; Takahashi et al., 1955).

An interesting pharmacological strategy is the modulation of GABAergic activity through a system involving a rapid uptake of GABA into presynaptic terminals and surrounding glial cells (Krogsgaard-Larsen, Falch, Larsson, & Schousboe, 1987; Thwaites, Basterfield, McCleave, Carter, & Simmons, 2000).

GABA is naturally present in many foods such as vegetables and fruits (Takayama & Ezura, 2015), especially in fermented products. Since GABA is considered a health-promoting functional compound, many dietary supplements and enriched foods have been developed to increase GABA intake. (Kook & Cho, 2013).

In Europe, the addition of GABA is considered a "dietary supplement" while in the United States it is considered a "food constituent". In Japan (Sanders, 1998), foods fortified with GABA can be denominated "foods for specified health use" (FOSHU), which have received great interest due to the increasing amounts of cases of hypertension in the general population, that is caused at least partially by the consumption of excessive amounts of sodium. Thus, many products have been developed to serve as vehicles for delivery of GABA (Becerra-Tomás et al., 2015). The enrichment of foods are based essentially on fermentation of substrates with GABA-producing bacteria (Inoue et al., 2003; Li, Qiu, Huang, & Cao, 2010). In this regard, dairy, soybean, kimchi and juice products could be used as potential GABA-delivery vehicles (Chang et al., 2009; Kim, Lee, Ji, Lee, & Hwang, 2009; Park & Oh, 2007; Seok et al., 2008). A

 

fermented milk product containing GABA was shown to have a blood pressure lowering effect in spontaneously hypertensive rats (Hayakawa et al., 2004) and in mildly hypertensive people (Inoue et al. 2003).

*Lactococcus lactis, i.e.,* the LAB model, has scantily been explored for this purpose. Thus, we chose a probiotic strain of *L. lactis* (NCDO2118 or NCDO) to optimize GABA production. Two concentrations of glutamate supplementation (0.5% and 5.0%) were evaluated. The bacterial culture with the highest concentration of glutamate showed a higher optical density in addition to the increase of GABA production over time; this increased growth could be attributed to the fact that glutamate can contribute favorably to the cell power generation. The alpha-ketoglutarate and succinate are intermediates of the Krebs cycle and hence related to cellular energy production. Glutamate can be deaminated to produce alpha-ketoglutarate. Alternatively, via "GABA shunt" glutamate may be decarboxylated to produce GABA; for this, succinic semialdehyde is converted to succinate, and is donated to an amino moiety that promotes alpha-ketoglutarate glutamate production. Alternatively, energy can be produced from glutamate since the enzymes are dependent on pyridoxal phosphate (Feehily & Karatzas, 2013; Waagepetersen, Sonnewald, & Schousboe, 2002).

After internalization of glutamate from the cell and GAD activity, the intracellular GABA is produced from the excess proton consumption in the midst of high-stress acid in the cytoplasm. The action of GAD is related to acid resistance in order to maintain cytoplasmic pH constant. A possible explanation for the observed slight increase in the pH at 48 hours, may be due to the maintenance of pH by GAD since the highest concentration of GABA production was observed at this time. Moreover in bacteria, GABA can be exported by the

 

presence of two glutamate\GABA Antiporters, contributing to cellular homeostasis.

After showing that the addition of glutamate significantly increased GABA production, the beneficial effect of the probiotic bacteria was evaluated in acute hypertension using a SHRSP rodent model hypertension.

*L. lactis* NCDO 2118 grown without glutamate is able to produce low concentrations of GABA. The hypertensive animals treated with this strain showed a progressive reduction in blood pressure with a maximum effect 2.5 hours after the start of pressure reduction. After this time, it took over approximately two more hours to restore hypertensive patterns of the animals tested. On the other hand, when the animals were treated with the same strain grown in high concentrations of glutamate that induced higher concentrations of GABA, a more pronounced effect on decreasing blood pressure was observed. Unlike what happened with the previous group, the reduction in mean arterial pressure was maintained until the final hours of the experiment. Although the results were favorable in this experiment while evaluating the beneficial effect of GABA in acute hypertension using the Biopac system, they were not observed when assessed using another analysis system. This conflicting data could be explained by the difference of recovery periods of the animal after anesthesia. Using the Biopac system for recording blood pressure values, the rats were submitted to the experimental treatment 24 hours after cannulation surgery, and in telemetry the experiments started 10 days after surgery, because telemetry surgery is a very complex procedure and animals requires a longer recuperation period. As TBE is an anesthetic that exerts sedative and hypnotic effects trough GABA system, a short period after anesthesia the GABA receptors could be more sensitive to

 

GABA (Krasowski & Harrison, 2000; Sauguet et al., 2013).

The mechanism by which GABA can exert its effect is unclear. Oral administration of GABA may be promoting any direct interaction in the brain, or their action is mediated through indirect ways such as by the enteric nervous system (ENS) (Boonstra et al., 2015). The ENS comprises a complex neural network located in the wall of the intestine, which is able to regulate intestine functions independently of the CNS (Auteri, Zizzo, & Serio, 2015). It is also known that following ingestion, GABA transepithelial transport across the intestinal wall can be mediated via a pH-dependent mechanism (H + / GABA symport) (Thwaites et al., 2000). The GABA receptor is distributed by ENS, both in the submucosa as neurons and in myenteric (Poulter, Singhal, Brown, & Krantis, 1999). Then, the increase of GABA level in the blood flow can explain the reduction effect on blood pressure observed in hypertensive rats.

## 5. Conclusion

In this work, the importance of the GABA produced by a probiotic LAB in the control of hypertension was suggested, showing that GABA has the potential to be used as a nutraceutical. However, more studies are needed to determine the GABA inflow tract, responsible for its beneficial effect. The optimization of probiotic bacteria producing GABA, is an interesting target for the development of foods enriched for therapeutic purposes.

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The authors declare that they have no competing interests.

 

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