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Probiotic Lactobacillus Reuteri Growth Improved under Fucoidan Exposure †

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Abstract: Allergy is the most common chronic disease in Europe and about 40% of the population suffers from it. The microbiota is closely linked to the immune system, being some probiotics characterized as beneficial contributing to reduce allergenic symptomatology. To ensure the predominance of beneficial probiotics on the gut microbiota, the use of prebiotics is very important. Among the most important prebiotics are those of marine origin, standing out the fucoidan, a polysaccharide from brown algae (*Phaeophyta*). The effect of fucoidan on *Lactobacillus reuteri* in vitro growth behaviour has been studied, using turbidimetry, plate count and statistical analysis. The fucoidan revealed a significant prebiotic effect increasing *L. reuteri* growth (≈2 Log_{10} cycles after 48 h exposure). The combination of the prebiotic effect of the fucoidan together with the direct action that it exerts on allergy and other health problems, make this molecule a promising nutraceutical for future.

Keywords: fucoidan; prebiotic; *Lactobacillus reuteri;* allergy

1. Introduction

Allergy is the most common chronic disease in Europe and about 40% of the population suffers from it. The microbiota plays a key role in the induction, control and function of the immune system. There is a symbiotic relationship between the microbiota and the immune system, so the immune system evolves as the complexity of the microbiota increases [1]. The gut microbiota (GM), although this is specific to each human being, consist mainly of four large phyla: Firmicutes (gram-positive), Bacteroids (gram-negative), Actinobacteria (gram-positive) and Proteobacteria (gram-negative) [2,3]. An imbalance (dysbiosis) in the percentages of each phylum translates into several changes and manifestations that alter the metabolic function of the immune system, which ends up generating different diseases, such as food allergies [3,4].

The diversity of GM in an individual changes from her/his birth to her/his maturity. The changes are influenced by genetics, epigenetics, and environmental factors. The GM differs, during the first months of life, in both composition and diversity in children with or without allergic diseases. Children allergic to nuts have a microbiota with an increase in *Bacteroides spp*. and children allergic to cow's milk show a decrease in the phylum Firmicutes [4]. In experimentation animals it is observed that an alteration of the microbiota affecting the families *Porphyromonadaceae*, *Lactobacillaceae*, *Rikenellaceae* and *Lachnospiraceae* may be behind the allergy to ovalbumin [5]. Li et al. [6] demonstrated that *Lactobacillus reuteri* is the most effective probiotic strain to reduce respiratory track inflammation since it reduces the total IgE and the production of Th2-associated inflammatory cytokines.

Prebiotics are typically polymers of carbohydrates that cannot be digested or absorbed by the human gut, although there are other compounds such as alcohols, enzymes, peptides, unsaturated

fatty acids, amino acids, or organic acids [7]. Algae are important sources of polysaccharides (PS) and oligosaccharides (OS) of great interest as prebiotics (alginates, carrageenans, exopolysaccharides, fucans and fucoids). Enzymes in the upper gastrointestinal tract do not break down PS from seaweed, so these PS can be used as food prebiotics and to enhance the growth of lactic acid bacteria such as *Lactobacillus spp.* and *Bifidobacterium spp.* [7,8]. Moreover, these PS from algae can be digested and fermented by GM to produce Short Chain Fatty Acids (SCFAs) that can improve the epithelial barrier function and the cardiovascular health [9].

A very interesting prebiotic is fucoidan, that includes a group of PS from the cell wall of some brown seaweeds and some invertebrate marine animals. The fucoidan from the seaweed *Fucus vesiculosus*, contains L-fucose and sulfate [10] and it can act as anticoagulant and anti-thrombotic, antimicrobial, antitumor and immunomodulatory, antioxidant, blood lipid reducer, anti-inflammatory, gastric protector, hepatoprotector and prebiotic [10,11]. As a result of its structure, fucoidan cannot be digested by gastric and pancreatic enzymes, so it reaches the small bowel unchanged where it can have a favourable effect on GM [12]. Fucoidan also promotes an enrichment in *Lactobacillus spp.* of pig colon [13] and stimulates the growth of *Bifidobacterium longum* and *B. bifidum* [7]. In mice, it stimulates the growth of bacteria of the family *Ruminococcaceae* and the genus *Lactobacillus* at the expense of reducing pathogenic bacteria of the genera *Peptococcus* and *Akkermansia* [14]. The production of SCFAs in pigs implies benefits such as protection against allergies [4,11,15].

We have evaluated the potential prebiotic effect of the fucoidan compound from the seaweed *Fucus vesiculosus* on the *Lactobacillus reuteri* growth to act against allergic symptoms.

2. Results

Absorbance measurements and bacterial counts allow to obtain the corresponding growth curve of the bacteria under study without the presence of fucoidan (control sample) during 48 h (see Figure 1). The latency phase of *L. reuteri* extends to 8 h of incubation. The exponential growth phase is observed from 8 to 24 h of incubation. The analysis of the bacterial growth when the Man Rogose Sharp [16] Broth (MRSB) medium is supplemented with fucoidan at two concentrations (100 and 2000 μg/mL) provides interesting results. Figure 2 reflects the results of the growth evolution ($Log_{10}(Nf)$ where Nf = is the number of living microorganisms at each studied time (in Colony Forming Units per milliliter (CFU/mL)) both in the control sample and in that supplemented with fucoidan, at different concentrations. Fucoidan has a prebiotic effect on *L. reuteri*. The prebiotic effect is maximum reaching 2.21 ± 0.19 cicles Log_{10} at 48 h of incubation.

A multifactorial analysis of variance is performed to check whether the concentration and the exposure times had a significant effect on the prebiotic nature of the fucoidan. The conclusion was that both the exposure time (p-value < 0.01) and the fucoidan concentration (p-value < 0.05) are factors with significant effect on the prebiotic nature of the fucoidan from *F. vesiculosus*. In the case of exposure time to fucoidan as a factor of influence over $Log_{10}(Nf)$, significant differences are observed between all measurements tested in the in vitro study, except exposure times between 24 and 26 h. This result allows us to state that the prebiotic effect occurs after 24 h of incubation and increases moderately at 28 h. In the case of the concentration of fucoidan as a factor of influence, significant differences are observed between the two concentrations and also respect the control sample. After 48 h of exposure even the fucoidan added at a relatively low concentration, 100 μg/mL, produces an increase over the control sample, of about 0.9 ± 0.6 cicles Log_{10}. In the case of 2000 μg/mL the increase is about 2.21 ± 0.19 cicles Log_{10}. It can be concluded that fucoidan shows a clear prebiotic effect the higher its concentration is in the range [100–2000] μg/mL.

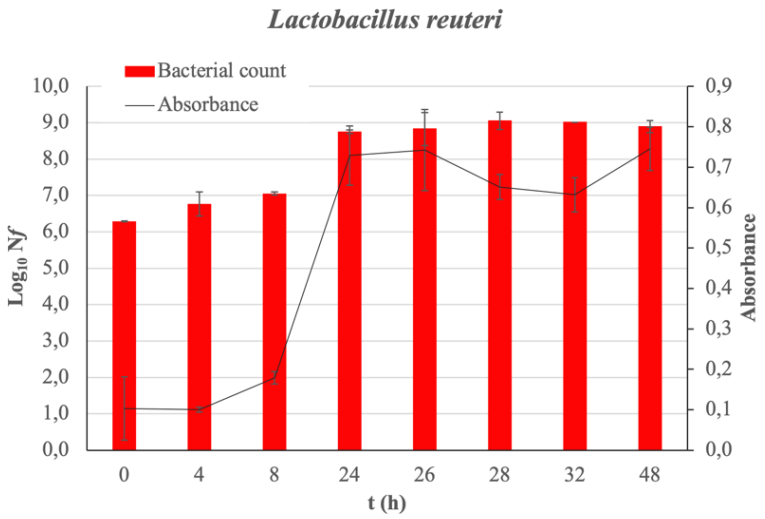

Figure 1. *L. reuteri* growth curve. The bar charts show the growth of bacteria in Log_{10}(N*f*) as function of incubation time. Absorbance measured as a function of incubation time is also plotted with a line graph. In all cases the standard deviation is represented.

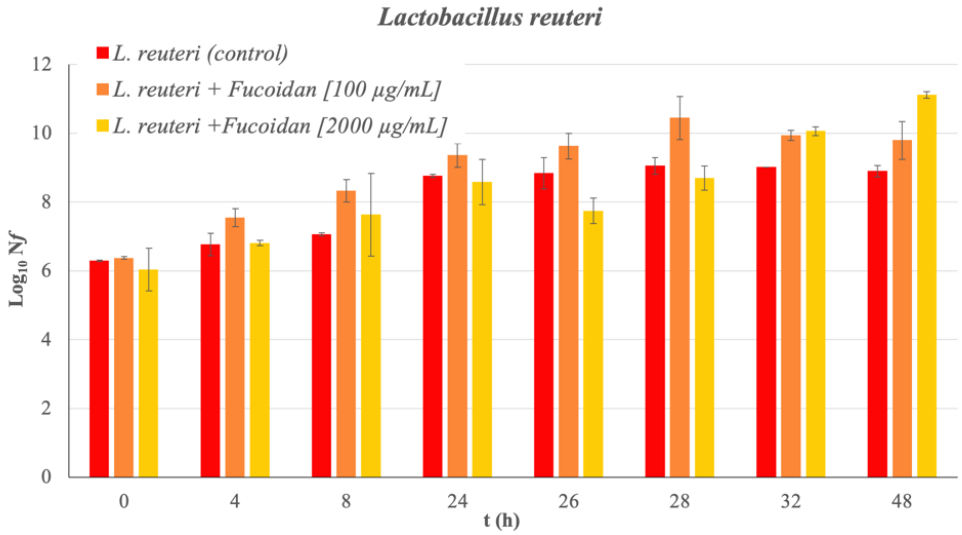

Figure 2. *L. reuteri* growth curve in different concentration of fucoidan. The bar charts show the growth of bacteria in Log_{10}(N*f*) as function of incubation time.

These two factors (exposure time and concentration) are crucial to define strategies for future usage of the compound in the formulation of prebiotic food and pharmaceutical products. The in vitro results obtained are promising and they suggest that fucoidan has a clear prebiotic effect on beneficial bacteria for the health and therefore it could help to improve allergic symptoms.

3. Discussion

The action mode of probiotic bacteria is multifactorial: production of direct inhibitory components on pathogenic bacteria, decrease at luminal pH through the production of SCFAs, competition for nutrients with pathogenic bacteria or modulation of the immune response [17]. Rosenfeldt et al. [18] demonstrated that a combination of *L. reuteri* and *L. rhamnosus*, administered as a probiotic, could help by improving atopic dermatitis in children and by decreasing the appearance of eczema. Published studies on the prebiotic nature of fucoidan from *F. vesiculosus* seaweed are limited. However, Wang et al. [19] observed in rats an increase in *Lactobacillus spp.* and *Bifidobacterium spp.* after ingestion of OS from brown seaweeds during 14 days at a concentration of 1% (weight/volume). Studies published by Palacios-Gorba et al. [20] demonstrated the antimicrobial

nature of the fucoidan from *F.vesiculosus*, *Undaria pinnatifida* and *Macrocystis pyrifera*, against one of the most worrying pathogens nowadays, the *Helicobacter pylori*, showing both bacteriostatic and bactericidal effects. Tannin et al. [52] determined that fucoidan increases galectin-9 levels. This increase is responsible of its antiallergic action. Niki et al. [21] determined that the galectin-9 secreted in blood by the fucoidan action, has the ability to attach to IgE, preventing the formation of the IgE-Antigen complex, by inducing an antiallergic effect. Fucoidan from *F. vesiculosus* is considered a "generally recognised as safe" ingredient and is beginning to be included in various products, such as dietary supplements to boost the immune system and as antioxidants.

4. Materials and Methods

Culture media and other reagents

The lactic acid bacteria were cultured in the liquid medium Man Rogose Sharp Broth (MRSB). The solid medium (MRSA) was prepared adding 2% agar. Dilutions were performed in sterile peptone water (1% w/V). Glycerol (20%) was used to prepare the frozen cultures as a cryoprotective agent. The culture media were supplied by the Conda-Pronadisa Laboratories (Madrid, Spain).

Bacterial strains

L. reuteri (CECT 925) was acquired from the Spanish Type Culture Collection (CECT) (Paterna, Spain). The culture was reactivated following the provider instructions and inoculated in a 20 mL flask of sterile MRSB. It was stirred during 3 h at 37 °C. Afterwards, 480 mL of sterile MRSB was added, and stirred again at 37 °C for 48 h [6]. The culture was then centrifuged (3000× *g*), 10 min, 25 °C. After removing the supernatant, the pellet was resuspended in sterile MRSB and subjected to a new centrifugation cycle. The pellet from this centrifugation was reserved and resuspended in 50 mL of a solution of MRSB and glycerol [80:20] (V/V). It was distributed in 2 mL cryovials, and these were stored frozen (−80 °C).

To determine the concentration of *L. reuteri* in the stored vials, three of them were taken and serial decimal dilutions were made in sterile peptone water (1% w/V). The seeding of the different dilutions was performed in triplicate on MRSA plates. The mean result obtained from the count was $(3.0 \pm 0.6) \times 10^9$ CFU/mL.

Fucoidans

Fucoidan lyophilisate from *F. vesiculosus* (95% purity) was purchased from (Sigma-Aldrich). The preparation of the stock solutions consisted of dissolving 500 mg of fucoidan powder in 50 mL of MRSB (concentration of 10 mg/mL). They were distributed in 10 mL aliquots in five sterile tubes and kept frozen (−80 °C) until the time of use.

Turbidimetry, growth curves and plate count

Measurements were performed on a spectrophotometer (T60U Spectrometer, PG Instruments Ltd.) at λ = 595 nm. The measured absorbance is correlated with the concentration of bacteria (Log10()). Precultures were prepared by diluting 100 μL of bacterial glycerin in 9.9 mL of MRSB. The solution was left to incubate at 28 °C during 72 h.

Control sample preparation consisted of mixing 20 μL of pre-culture and 19.98 mL of sterile MRSB. The fucoidan trial at 100 μg/mL was prepared by mixing 20 μL of pre-culture, 200 μL of fucoidan stock solution and 19.78 mL of sterile MRSB. The fucoidan trial at 2000 μg/mL was prepared by mixing 20 μL of the pre-culture, 4 × 103 μL of fucoidan stock solution and 15.98 mL of sterile MRSB. All cultures were incubated during 48 h in an oven at 28 °C. A sampling was performed periodically to monitor and record the growth of the bacteria, both in control sample and in fucoidan trials. Afterwards, 1 mL aliquots from the culture were taken in triplicate and the corresponding absorbances were measured. To obtain the bacterial concentration as a function of time, serial decimal dilutions and MRSA seeding were performed at different times during the incubation period. They were incubated in an oven at 28 °C during 48 h. Graphs were built to correlate concentration with time and absorbance.

Mathematical and statistical analysis

The growth curves and bar charts were built with the Excel program (Microsoft, Redmond, Washington, USA). An ANOVA (multifactorial analysis of variance) analysis was performed with

the Statgraphics 18 Centurion program (Statgraphics Technologies Inc., The Plains, Virginia, USA), in order to detect significant differences (p-value < 0.05) between bacterial growth (Log_{10} (Nf), Log_{10} (CFU/mL)) under the effect of the different studied factors (fucoidan concentration, exposure time, *Lactobacillus* strain).

5. Conclusions

Fucoidan from *Phaeophyceae* seaweed shows a prebiotic capacity on beneficial microorganisms, in improving allergic symptoms, and also it shows antimicrobial capacity against pathogens in the gastrointestinal tract. Fucoidan from *F. vesiculosus* shows, *in vitro*, a maximum prebiotic potential equivalent to 2.21 ± 0.19 Log_{10} cycles on *L. reuteri* (applied at a concentration of 2000 μg/mL). The combination of the prebiotic effect of fucoidan along with its direct action on allergies and other health problems, make this molecule a perfect candidate to become a nutraceutical, with a very promising future both in the pharmaceutical and food industry.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

GM	Gut microbiota
PS	Polysacchacarides
OS	Oligosaccharides
SCFAs	Short chain fatty acids
MRSB	Man Rogose Sharp Broth
MRSA	Man Rogose Sharp agar
CFU	Colony forming units

References

1. Belkaid, Y.; Harrison, O.J. Homeostatic Immunity and the Microbiota. *Immunity* **2017**, *46*, 562–576, doi:10.1016/j.immuni.2017.04.008.
2. Tinahones, F.J. La importancia de la microbiota en la obesidad. *Rev. Esp. Endocrinol. Pediatr.* **2017**, *8*, 16–20.
3. Icaza-Chávez, M. Microbiota intestinal en la salud y la enfermedad. *Rev. Gastroenterol. México* **2013**, *78*, 240–248, doi:10.1016/j.rgmx.2013.04.004.
4. Lee, K.H.; Song, Y.; Wu, W.; Yu, K.; Zhang, G. The gut microbiota, environmental factors, and links to the development of food allergy. *Clin. Mol. Allergy* **2020**, *18*, 5, doi:10.1186/s12948-020-00120-x.
5. Zhuang, L.; Chen, H.; Zhang, S.; Zhuang, J.; Li, Q.; Feng, Z. Intestinal Microbiota in Early Life and Its Implications on Childhood Health. *Genom. Proteom. Bioinform.* **2019**, *17*, 13–25, doi:10.1016/j.gpb.2018.10.002.
6. Li, L.; Fang, Z.; Liu, X.; Hu, W.; Lu, W.; Lee, Y.k.; Zhao, J.; Zhang, H.; Chen, W. *Lactobacillus reuteri* attenuated allergic inflammation induced by HDM in the mouse and modulated gut microbes. *PLoS ONE* **2020**, *15*, e0231865, doi:10.1371/journal.pone.0231865.
7. Zaporozhets, T.S.; Besednova, N.N.; Kuznetsova, T.A.; Zvyagintseva, T.N.; Makarenkova, I.D.; Kryzhanovsky, S.P.; Melnikov, V.G. The prebiotic potential of polysaccharides and extracts of seaweeds. *Russ. J. Mar. Biol.* **2014**, *40*, 1–9, doi:10.1134/S1063074014010106.
8. Hu, B.; Gong, Q.; Wang, Y.; Ma, Y.; Li, J.; Yu, W. Prebiotic effects of neoagaro-oligosaccharides prepared by enzymatic hydrolysis of agarose. *Anaerobe* **2006**, *12*, 260–266, doi:10.1016/j.anaerobe.2006.07.005.
9. Han, Z.L.; Yang, M.; Fu, X.D.; Chen, M.; Su, Q.; Zhao, Y.H.; Mou, H.J. Evaluation of Prebiotic Potential of Three Marine Algae Oligosaccharides from Enzymatic Hydrolysis. *Mar. Drugs* **2019**, *17*, 173, doi:10.3390/md17030173.
10. Li, B.; Lu, F.; Wei, X.; Zhao, R. Fucoidan: Structure and Bioactivity. *Molecules* **2008**, *13*, 1671–1695, doi:10.3390/molecules13081671.

11. de Jesus Raposo, M.F.; de Morais, A.M.M.B.; de Morais, R.M.S.C. Emergent Sources of Prebiotics: Seaweeds and Microalgae. *Mar. Drugs* **2016**, *14*, 27, doi:10.3390/md14020027.
12. Luthuli, S.; Wu, S.; Cheng, Y.; Zheng, X.; Wu, M.; Tong, H. Therapeutic Effects of Fucoidan: A Review on Recent Studies. *Mar. Drugs* **2019**, *17*, 487, doi:10.3390/md17090487.
13. Murphya, P.; Bello, F.D.; O'Doherty, J.; Arendt, E.K.; Sweeney, T.; Coffey, A. The effects of liquid versus spray-dried Laminaria digitata extract on selected bacterial groups in the piglet gastrointestinal tract (GIT) microbiota. *Anaerobe* **2013**, *21*, 1–8, doi:10.1016/j.anaerobe.2013.03.002.
14. Shang, Q.; Shan, X.; Cai, C.; Hao, J.; Li, G.; Yu, G. Dietary fucoidan modulates the gut microbiota in mice by increasing the abundance of *Lactobacillus* and *Ruminococcaceae. Food Funct.* **2016**, *7*, 3224–3232, doi:10.1039/C6FO00309E.
15. O'Doherty, J.; Dillon, S.; Figat, S.; Sweeney, T. The effects of lactose inclusion and seaweed extract derived from *Laminaria spp*. on performance, digestibility of diet components and microbial populations in newly weaned pigs. *Anim. Feed Sci. Technol.* **2010**, *157*, 173–180, doi:10.1016/j.anifeedsci.2010.03.004.
16. De Man, J.C.; Rogosa, M.; Sharpe, M.E. A medium for the cultivation of *Lactobacilli. J. Appl. Bacteriol.* **1960**, *23*, 130–135, doi:10.1111/j.1365-2672.1960.tb00188.x.
17. Tuohy, K.; Probert, H.; Smejkal, C.; Gibson, G. Using probiotics and prebiotics to improve gut health. *Drug Discov. Today* **2003**, *8*, 692–700, doi:10.1016/S1359-6446(03)02746-6.
18. Rosenfeldt, V.; Benfeldt, E.; Nielsen, S.D.; Michaelsen, K.F.; Jeppesen, D.L.; Valerius, N.H.; Paerregaard, A. Effect of probiotic *Lactobacillus* strains in children with atopic dermatitis. *J. Allergy Clin. Immunol.* **2003**, *111*, 389–395, doi:10.1067/mai.2003.389.
19. Wang, Y.; Han, F.; Hu, B.; Li, J.; Yu, W. In vivo prebiotic properties of alginate oligosaccharides prepared through enzymatic hydrolysis of alginate. *Nutr. Res.* **2006**, *26*, 597–603, doi:10.1016/j.nutres.2006.09.015.
20. Palacios-Gorba, C.; Pina, R.; Tortajada, M.; Jiménez-Belenguer, A.; Siguemoto, E.; Rodrigo, D.; Pina-Pérez, M. *Caenorhabditis elegans* as in vivo model to assess the FUCOIDAN bioactivity preventing *Helicobacter pylori* infection. *Food Funct.* **2020**, *11*, 4525–4534, doi:10.1039/D0FO00768D.
21. Niki, T.; Tsutsui, S.; Hirose, S.; Aradono, S.; Sugimoto, Y.; Takeshita, K.; Nishi, N.; Hirashima, M. Galectin-9 Is a High Affinity IgE-binding Lectin with Anti-allergic Effect by Blocking IgE-Antigen Complex Formation. *J. Biol. Chem.* **2009**, *284*, 32344–32352, doi:10.1074/jbc.M109.035196.