Unrevealing the inhibitory effect of Zn on presumptive *Lactobacillus* sp. RJ1: A Microscopic observation

Yaba Rosette Tanoé [1], Juhi Chauhan [2] Sandesh Telang [2], and Rakesh Kumar Sharma [3]*

[1] Faculty of Sciences, Friedrich-Alexander-Universität Erlangen-Nuernberg, 91054 Erlangen, Germany

[2] Department of Biosciences, Manipal University Jaipur, Jaipur – 303007, India

[3] Department of Life Sciences, Chhatrapati Shahu Ji Maharaj University, Kanpur – 208024, India

*Corresponding author, E-mail: rakeshsharma@csjmu.ac.in; r.18sharma@gmail.com; Orcid: 0000-0002-2826-1291

Received: 28-10-2024 Accepted:19-02-2024
Published: 01-10-2024

ABSTRACT
The study aims to evaluate the effect of Zn on a bacterial strain, which was isolated from the curd and biochemically characterized to be presumptive *Lactobacillus* sp. RJ1. Influence of varying concentrations of $ZnCl_2$ on the growth of isolated *Lactobacillus* sp. RJ1 was evaluated. Scanning electron microscopy, facilitated by a JEOL JSM-7610FPlus FESEM, provides detailed observations on biofilm formation. Elemental dispersion analysis of X-ray (EDX) provided elemental compositions of the sample surface. The research work further explored the interaction between the bacteria and cotton cloth fabric, which served as a surface for bacterial attachment. The findings validated *Lactobacillus* sp. RJ1's ability to form a well-structured biofilm and confirm the suitability of cotton cloth as a material for such experiments. Furthermore, the study uncovers the presence of different shapes of nanostructures on the surface of cotton threads primarily governed by the initial concentrations of $ZnCl_2$. At the 0.1M concentration of $ZnCl_2$ needle-shaped nanostructures ranging from 200 to 480nm in length were observed on the fiber surface. It was further observed that *Lactobacillus* sp. RJ1 could not form biofilm in the presence of $ZnCl_2$. Thus, it is suggested to use Zn resistant probiotic strain when co-administrating to treat intestinal dysenteries.

Keywords: Inhibitory effect; Zn nanostructures; *Lactobacillus* sp. RJ1

RESUMEN
El estudio tiene como objetivo evaluar el efecto del Zn en una cepa bacteriana, que fue aislada de la cuajada y caracterizada bioquímicamente como presunta *Lactobacillus* sp. RJ1. Influencia de diferentes concentraciones de $ZnCl_2$ en el crecimiento de Lactobacillus sp. Se evaluó RJ1. La microscopía electrónica de barrido, facilitada por un JEOL JSM-7610FPlus FESEM, proporciona observaciones detalladas sobre la formación de biopelículas. El análisis de dispersión elemental de rayos X (EDX) proporcionó composiciones elementales de la superficie de la muestra. El trabajo de investigación exploró más a fondo la interacción entre las bacterias y la tela de algodón, que servía como superficie para la adhesión de las bacterias. Los hallazgos validaron *Lactobacillus* sp. La capacidad de RJ1 para formar una biopelícula bien estructurada y confirma la idoneidad de la tela de algodón como material para tales experimentos. Además, el estudio revela la presencia de diferentes formas de nanoestructuras en la superficie de hilos de algodón gobernadas principalmente por las concentraciones iniciales de $ZnCl_2$. A una concentración de 0,1 M de $ZnCl_2$, se observaron nanoestructuras en forma de aguja de entre 200 y 480 nm de longitud en la superficie de la fibra. Se observó además que *Lactobacillus* sp. RJ1 no pudo formar biopelícula en presencia de $ZnCl_2$. Por lo tanto, se sugiere utilizar una cepa probiótica resistente al Zn cuando se coadministra para tratar la disentería intestinal.

INTRODUCTION

Zinc is an essential component in the treatment of diarrhea, which is especially important in young children. It reduces the duration and severity of diarrhea, boosts the immune system, improves intestinal health, enhances nutrient absorption, and complements oral rehydration therapy. Zinc supplementation has been recommended for the treatment of acute diarrhea in children by both the World Health Organization (WHO) and UNICEF because it is both cost-effective and supported by evidence [1]. Several studies have also suggested the use of zinc in the case of diarrhea and a lot of the pediatricians are using zinc and probiotic combination to treat such dysenteries [2].

Due to the distinct attributes and considerable importance, nanoparticles have emerged as a recent focal point of study in contemporary scientific research, representing a new and innovative area of investigation. ZnO nanoparticles have been reported to demonstrate beneficial effects on growth performance, intestinal morphology and microflora, and immunity, similar to the conventional ZnO [3]. In recent years, there has been a significant increase in scientific investigations focused on the fabrication of nanoparticles through the utilization of chemical, physical, and green or biological synthesis approaches. The use of green synthesis is increasingly substituting traditional physical and chemical processes due to its cost-effectiveness, environmentally safe, and non-toxic [4]. Different microbes such as *Bacillus cereus* [5], *Shewanella loichica* [6] have been utilized to synthesize inexpensive metallic nanoparticles, including silver, gold, zinc, and titanium. A range of analytical techniques are employed for the identification and characterization of nanoparticle synthesis. These techniques include UV-Vis Spectroscopy, Fourier transform infrared (FT-IR) spectroscopy, transmission electron microscopy (TEM), scanning electron microscopy (SEM), Energy Dispersive X-Ray (EDX), and X-ray diffraction (XRD). However, in the present study our aim was not no synthesize ZnO nanoparticles, or check their antimicrobial activity, but to evaluate the synergistic effect of Zn salt and bacterial strains, when co-administered simultaneously.

On the other hand, probiotics refer to living microorganisms that confer beneficial effects on the host cell. The predominant bacterial and fungal species frequently employed in probiotic formulations are *Lactobacillus*, *Bifidobacterium*, *Escherichia*, *Enterococcus*, *Bacillus*, *Streptococcus* and *Saccharomyces* [7]. Research findings indicate that probiotic strains have been associated with beneficial effects on digestive health, immune system enhancement, and decreased susceptibility to specific illnesses [8]. According to reports, *Lactobacillus* has demonstrated the ability to synthesize a range of nanoparticles, including gold, silver, and selenium and titanium dioxide [9–11]. Furthermore, a number of studies have demonstrated the efficacy of *Lactobacillus* in facilitating the production of ZnO nanoparticles [12,13]. Thus, *Lactobacillus* may help in synthesizing nanoparticles and is also a well-known biofilm form, which is primarily used in a probiotic preparation.

It is evident from several clinical trials that Zn and Probiotic combinations are giving variable results. Thus, the current research is focused on the evaluation of the influence of Zn on the growth and biofilm formation ability of isolated *Lactobacillus* sp. However, the isolated strain must meet the criteria that allow it to be considered as a potential probiotic and further application in the treatment of intestinal dysentery.

MATERIALS AND METHODS

Isolation, culturing of the bacterial strain

Bacterial culture of *Lactobacillus* sp. RJ1 used in this study was isolated from curd sample, which is a fermented product from a milk. Loopful curd sample was taken and streaked on selective media (MRS agar plates). The inoculated plates were incubated at 32°C for 4-5 days. The isolates were checked for catalase test and only negative samples were used after confirming the colony morphology and Gram's staining.

Characterization of isolated strain

For preliminary identification few tests were conducted; e.g. catalase test: a loopful of actively growing culture was taken on a glass slide and 3 % (v/v) H_2O_2 was added (2-3 drops) and observed for the formation of gas bubbles (O_2). For bright field microscopic examination and Gram's character the culture was processed as per the standard protocol [14] and observed under the under 400-1000X magnification.

Effect of various Zinc concentrations on the growth of *Lactobacillus* sp. RJ1

Bacterial culture was grown on MRS Broth at 32°C for 48 hours. Culture having 0.2 absorbance at 600nm was used as inoculum for the experiment. Different concentrations of $ZnCl_2$ were prepared i.e. 0.05 M, 0.1 M and 0.5 M along with blank (no $ZnCl_2$) in MRS broth tubes containing 2cm × 2cm cotton cloth piece (to form a biofilm) and sterilized in an autoclave. Cotton cloth was used as a matrix for the development of biofilm as it is porous, moisture retaining, biocompatible, economic, easily available and easy to handle at variable sizes. Three sets of each tube was prepared and 100 µl bacterial culture was inoculated after autoclaving and then incubated at 37°C for 5 days. A tube containing growth media and cotton cloth without bacteria was used a control.

Scanning electron Microscopy

A quick and easy method has been developed to study the microbial biofilm on cotton surface. Instead of the complex process for SEM observations, the cotton cloth piece form all the sets were removed from the tubes after 48 hrs of incubation, gently rinsed with distilled water and dried for 24 hrs at 50 °C. Completely dried cloth was used for the direct observations under the electron microscope. The cloth samples were removed after appropriate time interval and stored in a dry/airtight container till the observation. A small piece of the cloth was taken and placed on carbon tape. The specimen was coated with a thin gold layer through physical vapor deposition before subjecting to Scanning Electron Microscopy. The sample was then observed under different magnification using JEOL JSM-7610FPlus FESEM.

Elemental dispersion analysis of X-ray (EDX)

The FESEM JSM-7610F chamber was fitted with Energy Dispersive X-ray (EDS) detector for elemental analysis. EDX was used to determine the elemental composition on the dried cloth piece. The presence and absence of Zn were observed on the surface of all the samples.

analysis was carried out on two different CCA cables with 0.18, 0.24 and 0.25 mm diameters. Since the samples are already bi-metals, no surface coating is required for SEM analysis.To dehumidify samples, they were placed in the oven at 25°C for 2 hours. Then, CCA wire samples were emplaced on carbon stubs. Zeiss EVO-50 with Bruker-Axs XFlash 3001 SDD-EDX installed in Hacettepe University, Department of Geological Engineering, Ankara, Turkey has been used and operated under 15 kV accelerating voltage, 15 nA probe current. First of all, the samples have been visualized in BSE mode. Then, X-Ray chemical maps have been generated with at least 5 minutes of scanning. Line scan profiles along the zones of the corrosion have been applied. Qualitative chemical analyses have also been performed on the wire and the chemical substances.

RESULTS AND DISCUSSION

Biochemical characterization of isolated strain

Catalase test considered one of the significant test as *Lactobacillus* spp. do not produce catalase [15]. A catalase-positive strain *Bacillus subtilis* has been used as positive control. After the addition of hydrogen peroxide, bubbles of oxygen immediately appeared in *Bacillus subtilis*, while isolated *Lactobacillus* sp. RJ1, did not show any bubble formation.

Isolated *Lactobacillus* sp. RJ1 was Gram-positive. The bacteria showed purple colour and having rod-shaped structure. The bacterial cells were arranged as chain and sometimes in pairs (Figure 1). Based on these characteristics, the isolated strain seems a presumptive isolate of *Lactobacillus* sp.

Effect of various Zinc concentrations on the growth of *Lactobacillus* sp. RJ1

Cotton cloth fabric was used as a surface for the bacteria to develop the biofilm. The threads of the cloths provided a large surface area and allowed the attachment of the bacterial cells. As visible in the SEM images of the control (uninoculated) tube, the threads were intact and arranged, which could be used by the bacteria to form a biofilm (Figure 2).

Figure 1: Bright field microscopic view of Gram positive isolated *Lactobacillus* sp. RJ1 (magnification: 1000 X)

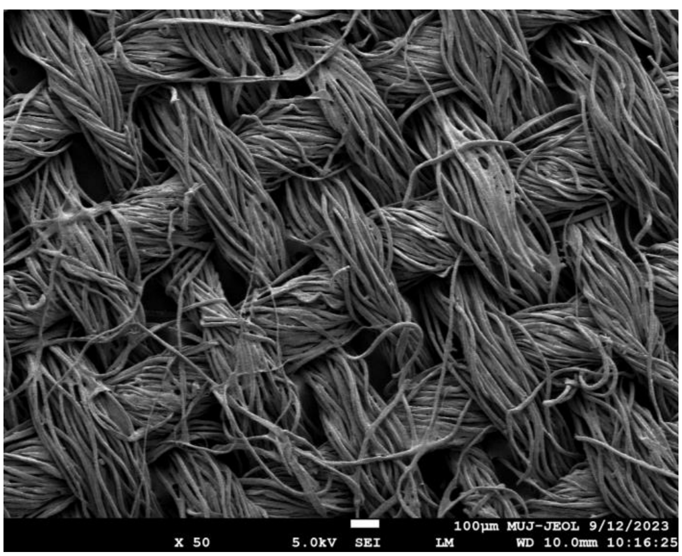

Figure 2 Scanning electron microscopic image of cotton cloth fabric showing well-arranged threads to provide surface for bacterial attachment at a magnification of 50 X and 5 kV.

The tube containing only bacteria *Lactobacillus* sp. RJ1 without Zn grow well and demonstrated well-arranged biofilm formation on the fabric surface. The observed cells showed typical lactobacillus cell morphology, rod-shaped, vertically dividing, arranged in the form of chain. The size of the cells was in the range of 1 µm to 2.5 µm in length and about 0.5 µm in width. The threads were fully covered by bacterial cell (Figure 3A-B). These observations confirmed the ability of *Lactobacillus* sp. RJ1 to form a well-structured biofilm and also the suitability of the cotton cloth as material to be used for such studies.

The presence of the bacterial cells was difficult to detect in all the samples as none of the Zn concentration could enhance or support the growth and biofilm formation by *Lactobacillus* sp. RJ1. On the other hand, different shapes of the ZnO nanoparticles were observed depending upon the initial concentration of $ZnCl_2$. It has been already reported that a precise and systematic control on the concentration of the precursor i.e. $ZnCl_2$ results to different shape and size of ZnO nanostructures [16]. On the other hand, different shapes of the ZnO nanoparticles were observed depending upon the initial concentration of $ZnCl_2$. Spherical nanostructures ranging from 50-200 nm were observed on the cloth having lowest concentration of $ZnCl_2$ i.e. 0.05M. The structures were aggregated and seems to be closely attached to the thread surface. On the other hand, no bacterial biofilm was observed (Figure 4A). At the 0.1M concentration of $ZnCl_2$, needle-shaped structures ranging from 250nm to 480nm in length and about 55nm in width were detected only at the concentration of 0.1M of Zn (Figure 4B). Zinc oxide nanostructures of different shapes including needle (nanorods of 6 - 7 µm in length and 50-100 nm of width) flowers, pencil and square shaped structures have also been synthesized and reported, which might be used for the potential applications in variety of fields [17]. In the present study, as observed at the highest concentration of $ZnCl_2$ cubes or square shaped nanostructures were observed, which were also closely associated to the surface (Figure 4C).

As compared to other two concentrations of $ZnCl_2$, in the case of 0.1M well-structured and much more regulated shape and size of the nanostructures was observed. However, the other structures were showing agglomeration and closely adhered to the surface, while the needle structures were sharp and clear as observed under the SEM (Figure 5)

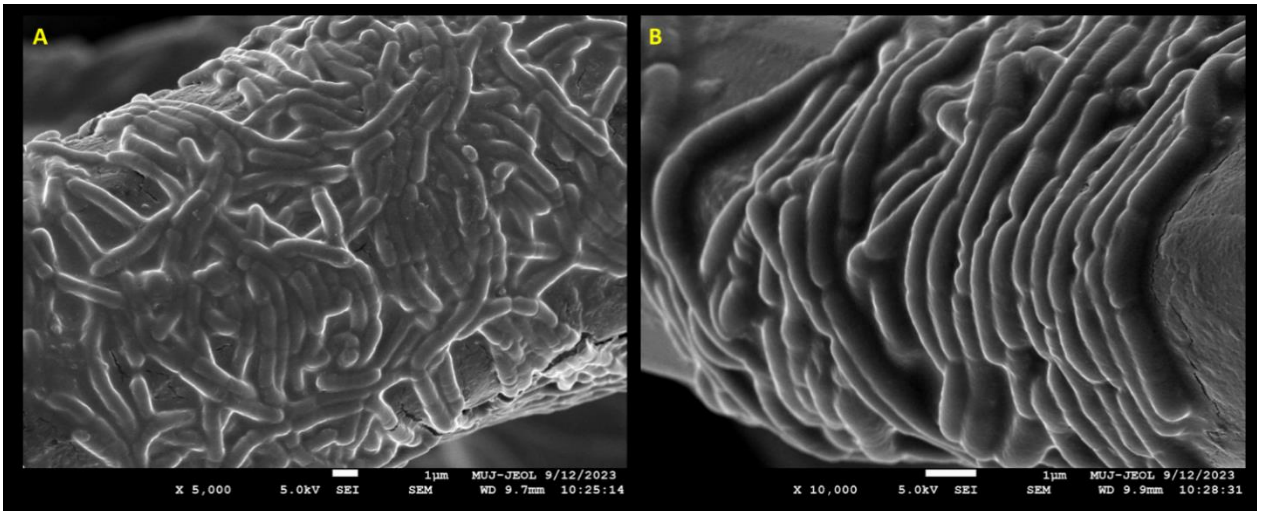

Figure 3 Scanning electron microscopic image of *Lactobacillus* sp. RJ1 growth on the cotton threads and formation of biofilm; (A) arrangements of cells at 5000X and 5 kV, (B) arrangements of cells at 10000X and 5 kV.

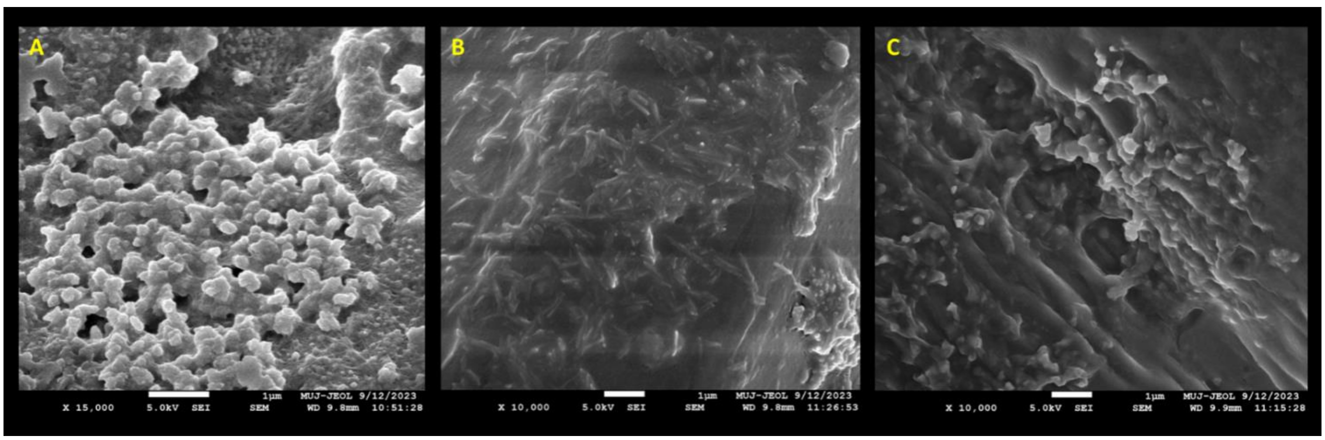

Figure 4 Visible nanostructures on the surface of cotton thread at different initial concentration of $ZnCl_2$; (A) 0.05M at 15000X and 5 kV, (B) 0.1M at 10000X and 5 kV, and (C) 0.05M at 10000X and 5 kV

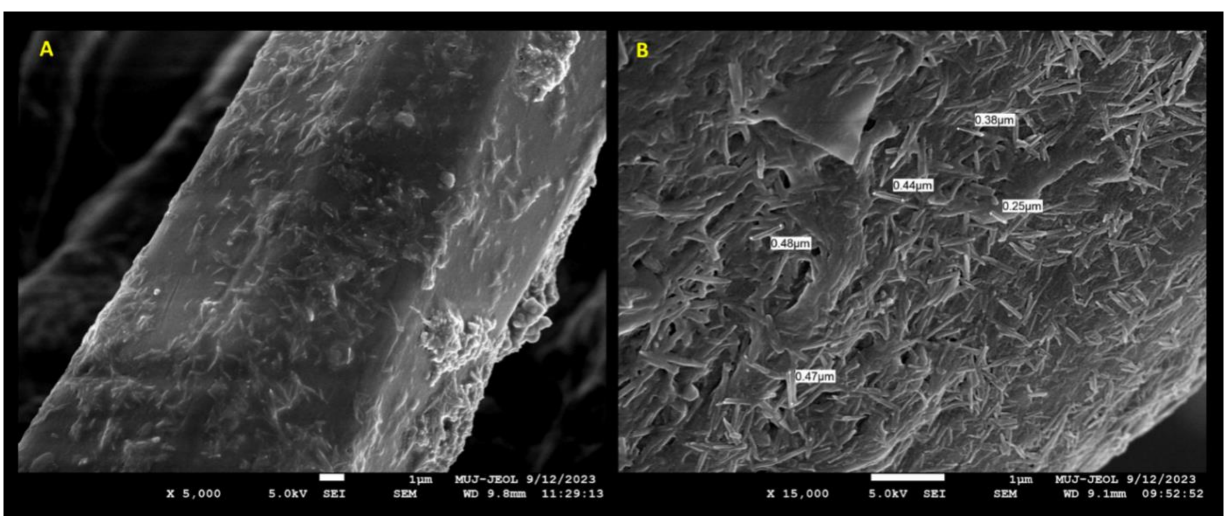

Figure 5 Scanning electron microscopic images showing (A) Needle shape nanostructures on the cotton thread at 5000 X and 5 kV (B) Size of the needles ranged from 200 – 480 nm in length as observed at 15000 X and 5 kV

EDX analysis of the samples revealed that only C and O along with come concentration of Na was detected in the control sample and there was no other significant peak was detected. On the other hand, Zn and Cl peaks were detected in the samples, which were inoculated with $ZnCl_2$ (Figure 6). The Zn and Cl concentration were also in the same range as used initially. Thus EDX analysis confirmed the presence of only one metal i.e. Zn in all the samples except the control. Based on the above observations it may be concluded that $ZnCl_2$ might act as precursor to synthesise nanostructures. However, this was not the primary aim of the study, these structures of different size and shape are primarily governed by the initial concentration of the $ZnCl_2$ salt. The reaction might have lead by the bacterial presence, however, no significant number of bacterial cells were observed in any of the sample except the control one. It also possible the cotton fibres might be involve in the synthesis of such kind of nanostructures, which were used as a template and allow the surface area for nanostructure synthesis [18].

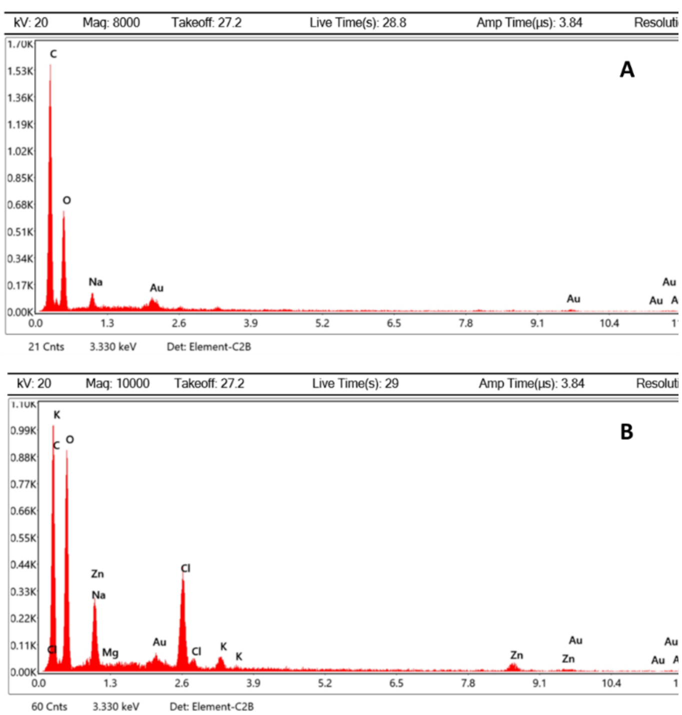

Figure 6 EDX analysis showing the presence of different elements on cotton fibre surface: (A) C and O on the control and (B) C, O, K, Zn and Cl along with Na in the 0.1M $ZnCl_2$ containing sample

Thus, it was clear that either or both the possible mechanism for nanostructure synthesis might be involved in the present study. However, in any case, the bacteria could not get the available surface for the attachment to develop a biofilm. The same was observed in the present study. Further, to understand the synergistic effect of Zn and probiotic, this point must be taken care of. If the nanostructures are developed no bacterial biofilm is formed and probiotic may not function as per its desired application.

In earlier study, it was observed that infants in the Probiotic Receiving Group had a higher rate of diarrhea persistence on the third and fourth days of admission compared to the Zinc Receiving Group. As a result, the study concluded that zinc supplementation at a 20 mg dose was more effective and associated with fewer complications than probiotics in treating diarrhea in infants [19]. However, the study did not report anything on the combination of both. Similarly, another study was not able to demonstrate a significant clinical benefit of the probiotic *Bacillus clausii* and zinc supplement therapy to treat acute diarrhea in Indian children [20]. Thus, the studies have varied results but, the combination of Zn and probiotic is not completely ensured as the Zn maybe toxic to most of the available strains and in some cases the mechanisms are also unknown. Most of the probiotic bacteria are sensitive to zinc and most of them cannot grow in the presence of zinc. It was further, suggested to use zinc tolerant probiotic streams as a combination to get the highest benefit of the probiotic properties of the bacteria. In the present study, it was observed that the recommended amount of the zinc may not suitable for the growth of probiotic bacteria, which was used in the present study. There are several factors, which can negatively influence the growth of probiotic bacteria, however, one of the present observation reveals the production or synthesis of zinc nanoparticles, which might be toxic for the bacteria to grow and may not allow them to form a proper biofilm. Further, the molecular identification and characterization of the isolated strain as probiotic may be carried out to further endorse its clinical applications based on the resistance to gastrointestinal transit, adhesion to intestinal epithelial cells, bactericidal activity and susceptibility to antibiotics.

CONCLUSION

In conclusion, this study unveils few essential insights into the relationship between zinc, an isolated strain i.e. presumptive *Lactobacillus* sp. RJ1, and the formation of biofilms. The ability of the isolated strain to form well-structured biofilms holds promise for its potential application in the treatment of intestinal dysenteries. The use of cotton cloth as a support material for bacterial attachment has been validated, opening new avenues for biofilm-related studies. However, the diverse outcomes observed during the present study including the combination of zinc and bacteria demonstrated the inhibitory effect of zinc on the growth of *Lactobacillus* sp. RJ1, which might be the non-availability of the surface to form a biofilm as Zn nanostructures were observed on the surface of cotton thread. The presence of different nanostructures on the cotton thread's surface, influenced by different initial concentrations of $ZnCl_2$, highlights the multifaceted nature of this interaction. However, these findings may not confirm the administration of Zn salt and probiotics ration, which may be further explored before clinical trials.

ACKNOWLEDGEMENT

YRT is thankful to the IAESTE for Providing assistance in international movement. Authors are thankful to Manipal University Jaipur for providing SEM and EDX facility.

CONFLICT OF INTEREST

The author declares no conflict of interest.

REFERENCES

[1] Salvatore, S., Hauser, B., Devreker, T., Vieira, M.C., Luini, C., Arrigo, S. et al. (2007) Probiotics and zinc in acute infectious gastroenteritis in children: are they effective? *Nutrition* **23**:498–506. https://doi.org/10.1016/j.nut.2007.03.008

[2] Shamir, R., Makhoul, I.R., Etzioni, A. and Shehadeh, N. (2005) Evaluation of a Diet Containing Probiotics and Zinc for the Treatment of Mild Diarrheal Illness in Children Younger Than One Year of Age. *Journal of the American College of Nutrition* **24**:370–5. https://doi.org/10.1080/07315724.2005.10719487

[3] Pei, X., Xiao, Z., Liu, L., Wang, G., Tao, W., Wang, M. et al. (2019) Effects of dietary zinc oxide nanoparticles supplementation on growth performance, zinc status, intestinal morphology, microflora population, and immune response in weaned pigs. *Journal of the Science of Food and Agriculture* **99**:1366–74. https://doi.org/10.1002/jsfa.9312

[4] Bloch, K., Pardesi, K., Satriano, C. and Ghosh, S. (2021) Bacteriogenic Platinum Nanoparticles for Application in Nanomedicine. *Frontiers in Chemistry* **9**. https://doi.org/10.3389/fchem.2021.624344

[5] Sunkar, S. and Nachiyar, C.V. (2012) Biogenesis of antibacterial silver nanoparticles using the endophytic bacterium Bacillus cereus isolated from Garcinia xanthochymus. *Asian Pacific Journal of Tropical Biomedicine* China. **2**:953–9. https://doi.org/10.1016/S2221-1691(13)60006-4

[6] Lv, Q., Zhang, B., Xing, X., Zhao, Y., Cai, R., Wang, W. et al. (2018) Biosynthesis of copper nanoparticles using Shewanella loihica PV-4 with antibacterial activity: Novel approach and mechanisms investigation. *Journal of Hazardous Materials* **347**:141–9. https://doi.org/https://doi.org/10.1016/j.jhazmat.2017.12.070

[7] Fijan, S. (2014) Microorganisms with claimed probiotic properties: an overview of recent literature. *International Journal of Environmental Research and Public Health* Switzerland. **11**:4745–67. https://doi.org/10.3390/ijerph110504745

[8] Das, T.K., Pradhan, S., Chakrabarti, S., Mondal, K.C. and Ghosh, K. (2022) Current status of probiotic and related health benefits. *Applied Food Research* **2**:100185. https://doi.org/https://doi.org/10.1016/j.afres.2022.100185

[9] Jha, A.K. and Prasad, K. (2010) Biosynthesis of metal and oxide nanoparticles using Lactobacilli from yoghurt and probiotic spore tablets. *Biotechnology Journal* **5**:285–91. https://doi.org/https://doi.org/10.1002/biot.200900221

[10] Kato, Y., Yoshimura, E. and Suzuki, M. (2019) Synthesis of Gold Nanoparticles by Extracellular Components of Lactobacillus casei. *ChemistrySelect* **4**:7331–7. https://doi.org/https://doi.org/10.1002/slct.201901046

[11] Rajasree, R.S.R. (2015) Extracellular biosynthesis of Selenium nanoparticles using some species of Lactobacillus. NISCAIR-CSIR, India.

[12] El-Sayed, H.S., El-Sayed, S.M. and Youssef, A.M. (2021) Novel approach for biosynthesizing of zinc oxide nanoparticles using Lactobacillus gasseri and their influence on microbiological, chemical, sensory properties of integrated yogurt. *Food Chemistry* **365**:130513. https://doi.org/https://doi.org/10.1016/j.foodchem.2021.130513

[13] Mohd Yusof, H., Abdul Rahman, N., Mohamad, R., Zaidan, U.H. and Samsudin, A.A. (2020) Biosynthesis of zinc oxide nanoparticles by cell-biomass and

supernatant of Lactobacillus plantarum TA4 and its antibacterial and biocompatibility properties. *Scientific Reports* **10**:19996. https://doi.org/10.1038/s41598-020-76402-w

[14] Coico, R. (2006) Gram Staining. *Current Protocols in Microbiology* **00**. https://doi.org/10.1002/9780471729259.mca03cs00

[15] Salvetti, E., Torriani, S. and Felis, G.E. (2012) The Genus Lactobacillus: A Taxonomic Update. *Probiotics and Antimicrobial Proteins* **4**:217–26. https://doi.org/10.1007/s12602-012-9117-8

[16] Panigrahy, B., Aslam, M., Misra, D.S. and Bahadur, D. (2009) Polymer-mediated shape-selective synthesis of ZnO nanostructures using a single-step aqueous approach. *CrystEngComm* **11**:1920. https://doi.org/10.1039/b904833m

[17] Wahab, R., Kim, Y.-S. and Shin, H.-S. (2011) Fabrication, characterization and growth mechanism of heterostructured zinc oxide nanostructures via solution method. *Current Applied Physics* **11**:334–40. https://doi.org/10.1016/j.cap.2010.07.030

[18] Shaheen, T.I., El-Naggar, M.E., Abdelgawad, A.M. and Hebeish, A. (2016) Durable antibacterial and UV protections of in situ synthesized zinc oxide nanoparticles onto cotton fabrics. *International Journal of Biological Macromolecules* **83**:426–32. https://doi.org/10.1016/j.ijbiomac.2015.11.003

[19] Ahmadipour, S., Mohsenzadeh, A., Alimadadi, H., Salehnia, M. and Fallahi, A. (2019) Treating Viral Diarrhea in Children by Probiotic and Zinc Supplements. *Pediatric Gastroenterology, Hepatology & Nutrition* **22**:162. https://doi.org/10.5223/pghn.2019.22.2.162

[20] Lahiri, K.R., Singh, R., Apte, M., Patil, M., Taksande, A., Varona, R. et al. (2022) Efficacy and safety of Bacillus clausii (O/C, N/R, SIN, T) probiotic combined with oral rehydration therapy (ORT) and zinc in acute diarrhea in children: a randomized, double-blind, placebo-controlled study in India. *Tropical Diseases, Travel Medicine and Vaccines* **8**:9. https://doi.org/10.1186/s40794-022-00166-6