Effect of 17β-estradiol on a Human Vaginal Lactobacillus Crispatus Strain

Maximilien Clabaut
University of Rouen Normandy
Amandine Suet
French National Centre for Scientific Research
Pierre-Jean Racine
University of Rouen Normandy
Ali Tahrioui
University of Rouen Normandy
Julien Verdon
University of Poitiers
Magalie Barreau
University of Rouen Normandy
Olivier Maillot
University of Rouen Normandy
Agathe Le Tirant
Sequens Cosmetics
Madina Karsybayeva
Remedials Laboratory
Coralie Kremser
Gymopharm
Gérard Redziniak
Cosmetic Inventions
Cécile Duclairoir-Poc
University of Rouen Normandy
Chantal Pichon
French National Centre for Scientific Research
Sylvie Chevalier
University of Rouen Normandy
Marc Feuilloley (✉ marc.feuilloley@univ-rouen.fr)
University of Rouen Normandy

Research Article

Keywords: Host-bacteria communication, Vaginal homeostasis, Bacterial adhesion, Bacterial virulence, Estrogens, Microbial endocrinology

Posted Date: December 8th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-114432/v1

Version of Record: A version of this preprint was published at Scientific Reports on March 30th, 2021. See the published version at https://doi.org/10.1038/s41598-021-86628-x.

Abstract

Lactobacillis and estrogens are essential in vaginal homeostasis. In the present study, we investigated the potential direct effect of 17β-estradiol on a vaginal strain of *Lactobacillus cristapus*, the major bacterial species of the vaginal microbiota. 17β-estradiol (10^{-6} to 10^{-10} M) has no effect on the growth of *L. crispatus* but markedly affects its membrane dynamic. This effect appears adaptative and coherent with a signal transduction process. The surface polarity and aggregation potential of the bacteria remains unchanged after exposure to 17β-estradiol but its mean size is significantly reduced. 17β-estradiol also promotes biosurfactant production by *L. crispatus* and adhesion to vaginal VK2/E6E7 cells. Conversely, the biofilm formation activity of the bacterium is marginally affected. A potential 17β-estradiol binding site was identified by bioinformatics in *L. crispatus* as the membrane lipid rafts associated SPFH domain containing protein. Taken together, this study reveals that 17β-estradiol can exert direct effects on *L. crispatus*. This process might be of importance in the vaginal environment physiology by promoting adhesion of *Lactobacilli* to the mucosa and providing protection against pathogens.

Key Points

We demonstrate that 17β-estradiol can exert direct effects on a vaginal strain of *Lactobacillus crispatus*, particularly membrane dynamic, biosurfactant production and adhesion to vaginal cells. This process might be of importance in the vaginal physiology by promoting adhesion of *Lactobacilli* to the mucosa.

Introduction

The human microbiota is a dynamic structure, which differs at the levels of organs and populations[1]. It is in constant evolution in response to local and environmental factors[1-3]. The vaginal microbiota, one of the more variable, evolves regarding women physiology[4]. In young prepubescent girls, it is essentially composed of microorganisms from cutaneous and fecal origins[5]. After puberty, the pH of the vaginal environment decreases, mostly because of colonization by acid producers[6] such as *Lactobacilli*, including *L. crispatus, L. iners, L. jensenii* and/or *L. gasseri* that becomes the predominant bacteria of this microbiota at this physiological stage[7-8]. In women of reproductive age, the vaginal microbiota composition remains quite stable, until menopause. At this stage, the *Lactobacilli* population decreases and as a result the local vaginal pH returns to neutrality[6], leading *Staphylococci* and other skin microorganisms to colonize the vaginal mucosa[9-10]. Finally, the composition of post-menopause vaginal microbiota becomes almost the same as the cutaneous microbiota. These events are frequently associated to gynecological troubles, including itching, inflammation and even infections[11], since *Lactobacilli* naturally provide protection against pathogens. This protection is due to the large secretory activity of *Lactobacilli,* which produce acids, responsible of vaginal pH decrease[6], hydrogen peroxide[6,12], exoenzymes, bacteriocins and surfactant[13-15]. For those reasons, different strategies aiming at re-implanting *Lactobacilli* in post-menopause women have been developed, either through introduction of

exogenous bacteria (probiotics), addition of nutriments (prebiotics) or a combination of both (symbiotics)[16]. The prevalence of *Lactobacilli* in the vaginal microbiota is of importance to protect women from gynecological disorders and the question relies then on understanding why *Lactobacilli* population decreases in post-menopaused women. Variations of pH seems not to be the major factor resulting in *Lactobacilli* decrease since even when the vaginal medium is artificially re-acidified, *Lactobacilli* remain unable to stabilize and exert efficiently a protective function over a long time[17]. One reason could be the important decrease of estrogens production observed during menopause, compared to reproductive-aged women, which affects the dynamic and lubrication of the vaginal mucosa[11,18]. However, another cause could be related to the potential direct effect of estrogens on *Lactobacilli*.

Since the end of the XXth century, it has been demonstrated that bacteria can sense and adapt to host factors[19]. The effects of peptidic hormones and classical neurotransmitters have been particularly studied[20-21]. Our knowledge on the impact of steroid hormones is however much more limited. In skin, testosterone has been shown to promote indirectly *Cutibacterium acnes* development by increasing surface lipids availability[22] and estrogens appear to modulate the composition and metabolic activity of the gut microbiota[23]. Direct effects of estradiol on bacteria were also reported. In *Agrobacterium tumefaciens* and *Pseudomonas aeruginosa*, estradiol is interfering with virulence expression through inhibiting inter-bacterial communication[24-25]. In addition, estradiol stimulates the growth and regulates biofilm formation, coaggregation and polysaccharide production in *Prevotella intermedia*[26]. An effect of estradiol on *Staphylococcus epidermidis* growth and biofilm formation was also reported[27]. Nonetheless, to our knowledge, the effects of estradiol on *Lactobacilli* have not been investigated until now.

In the present study, the effects of estradiol on a vaginal strain of *L. crispatus*, whose genome was recently sequenced[28], was investigated through physiological, physicochemical, morphological, cell binding assay and bioinformatics approaches. Our results demonstrate that estradiol can affect the *Lactobacilli* physiology, suggesting that estradiol could play a key role in their implantation onto the vaginal mucosa.

Results

17β-estradiol alters *L. crispatus* CIP104459 membrane fluidity. Preliminary studies showed that 17β-estradiol at 10^{-6}, 10^{-8} and 10^{-10} M did not affect *L. crispatus* CIP104459 growth (**Suppl Fig. 1**). As an amphiphilic molecule, 17β-estradiol can integrate into phospholipid membranes and affect their fluidity. To investigate the effect of 17β-estradiol on *L. crispatus* CIP104459 membrane fluidity, fluorescence anisotropy analysis was performed as previously described[29]. Since anisotropy reflects the degree of organization of the bacterial membrane, a decrease of this index indicates a less organized phospholipid bilayer and thus an increased membrane fluidity. Bacteria were grown for 18 h in absence or presence of 17β-estradiol at 10^{-6}, 10^{-8} and 10^{-10} M before the anisotropy index was measured. As shown on **Fig. 1A**, 17β-estradiol decreased fluorescence anisotropy. This effect was statistically significant with 17β-estradiol at 10^{-6} and 10^{-10} M. At 10^{-8} M, the difference in regard of the control value was less important

and non-significant. Altogether, these data show that 17β-estradiol increased *L. crispatus* CIP104459 membrane fluidity. This could be explained either by integration of 17β-estradiol in the bacterial membrane or by an adaptive response of the bacteria after 17β-estradiol sensing as an environmental signal. To get further insights into the mechanism leading to this effect, bacteria were grown for 6, 12 or 24 h in MRS medium before 17β-estradiol was added, and the anisotropy was followed every 15 min for 3 h (**Fig. 1B, 1C and 1D**, respectively). Remarkably, the fluorescence anisotropy of control bacteria collected after 6 h of culture, and to a lesser extent for 12 h, tends to spontaneously increase. No variation was detected when bacteria were grown to their stationary phase (24 h) suggesting that they reached a steady step of membrane organization. When 6 h culture bacteria were exposed to 17β-estradiol, anisotropy values were noisy, probably because of membrane instability, but evolved over 3 h as the control. The signal was more stable with bacteria grown for 12 and 24 h before treatment with 17β-estradiol and no difference with the control was noted. If the effect of 17β-estradiol was due to non-specific interaction with the membrane, the process should be rapid and associated to an immediate decrease of anisotropy. Moreover, whereas limited variations of membrane fluidity were noted using 6 h old cultures, they were totally absent using stationary-phase grown cells. Then, these data indicate that the increased membrane fluidity of *L. crispatus* CIP104459 observed after exposure to 17β-estradiol from the beginning to the end of the culture, probably results from an adaptative process. The latter exposition condition was used in the subsequent assays.

17β-estradiol marginally affects the polarity and Lewis acid/base surface properties of *L. crispatus* CIP104459. Considering the effect of 17β-estradiol on membrane fluidity, the surface polarity of *L. crispatus* CIP104459 was studied by the microbial affinity to solvents (MATS) assay. This bacterium shows an overall limited affinity to solvents, particularly to apolar ones such as decane and hexadecane (mean value 12 ± 2.19 % and 12 ± 1.36 %, respectively), whereas the affinity to more polar solvents such as ethyl acetate and chloroform was higher (24 ± 1.21 % and 32 ± 1.62 %, respectively) (**Figs. 2A, 2B, 2C and 2D**). These results indicate that *L. crispatus* CIP104459 has a hydrophilic surface. Exposure to 17β-estradiol tends to induce a limited increase of affinity to chloroform and a parallel decrease of affinity to the three other solvents. Only the decrease of affinity to ethyl acetate 10^{-8} M was statistically significant ($p< 0.05$). Evaluation of the Lewis acid/base ratio using the two solvent couples hexadecane/chloroform or decane/ethyl acetate couples revealed that all bacteria exposed to 17β-estradiol were showing an evolution of their electron donor/acceptor behavior. While the untreated bacteria surface displayed acido-basic characteristics, 17β-estradiol treated bacteria showed a basic surface (**Fig. 2E**). However, this evolution was marginal and the difference was only significant for the couple hexadecane/chloroform with bacteria exposed to 17β-estradiol 10^{-10} M.

17β-estradiol alters *L. crispatus* CIP104459 morphology but not its aggregation phenotype. Since membrane fluidity may affect cell morphology or aggregation, we next assessed the effects of 17β-estradiol on *L. crispatus* CIP104459 on these phenotypes. To investigate the aggregation phenotype at a macroscopic level, we first performed a “sedimentation” technic as previously described by Vandevoorde *et al.*[30]. No effect of 17β-estradiol on the aggregation potential of *L. crispatus* CIP104459 was observed

in these conditions. The percentage of aggregation reached a mean value of 66.6 ± 1.6 % in control bacteria and of 65.3 ± 2.2, 62.9 ± 1.3, and 66.4 ± 1.2 % in bacteria grown in the presence of 17β-estradiol 10^{-6}, 10^{-8} and 10^{-10} M, respectively (**Suppl Fig. 2**). The effect of 17β-estradiol on *L. crispatus* CIP104459 was further assessed by flow cytometry, an approach that provides informations regarding cell aggregation, mean size, and structure heterogeneity (granularity). These informations are respectively given by the forward scattering fraction (FSC) of the light and by the light scattered laterally, also designated as side scatter (SSC)[31]. As illustrated by plotting the FSC and SSC values of *L. crispatus* CIP104459, in FSC (X axis), the maximal size of detected events (in red) was decreased after exposure to 17β-estradiol whereas the signal corresponding to small size events (in blue) increased (**Fig. 3A**). This was particularly clear for bacteria exposed to 10^{-6} and 10^{-8} M of 17β-estradiol, and to a lesser extent to 10^{-10} M of 17β-estradiol, although small-sized particles remained predominant compared to the control condition. By contrast, the surface heterogeneity (granularity) of the bacteria, corresponding to the SCC values (Y axis), was not modified. The difference of FSC values between control and 17β-estradiol treated bacteria was not significant, probably as this signal combines two informations, the aggregation degree of the bacteria and their mean size. Altogether, these data suggest that 17β-estradiol affects *L. crispatus* CIP104459 morphology. Scanning electron microscopy (SEM) observations of cells revealed that 17β-estradiol had no detectable effect on aggregates formation (**Fig. 3B**). Measurements of cells length on SEM images led to classify *L. crispatus* CIP104459 from small (1 to 2 µm) to large (4 to 9 µm) sized bacteria. Remarkably, 17β-estradiol treatment at 10^{-8} and 10^{-10} M led to increase significantly the small sized bacteria (p <0.001) while decreasing the larger ones (p < 0.01) (**Fig. 3C**).

17β-estradiol promotes biosurfactant production by *L. crispatus* CIP104459. Biosurfactants are surface-active molecules reducing the air/water surface tension to low levels, which can for some of them insert into bacterial membranes and modify membrane fluidity[32]. As shown in **Fig. 4A**, cultures of *L. crispatus* CIP104459 exposed to 17β-estradiol (10^{-6} and 10^{-8} M) over 48 h and layered on MRS-agar were spreading and flowing over the Petri dishes suggesting biosurfactant production. When the bacterial mats of microorganisms exposed or not to 17β-estradiol (10^{-6}, 10^{-8} and 10^{-10} M) was submitted to extraction in water, solutions obtained showed different behavior when they were tested by the sessile drop technique on polystyrene surface (**Fig. 4B**). Controls made using 17β-estradiol 10^{-6} to 10^{-10} M alone in water showed that the steroid alone had no effect (*data not shown*). In comparison to pure water (control), the drops of solution from bacteria exposed to 17β-estradiol were flattened, particularly when they were extracted from bacterial mat treated with 17β-estradiol 10^{-6} M. This decrease of the contact angle of the liquid with the surface indicates a reduction of the surface tension and therefore the presence of biosurfactant. To ascertain the surface tension reduction, the pendant drop method was used and the mean surface tension of the solution was calculated from the modeling of the shape of hanging drop air visualized by camera. A dose related decrease of the surface tension of solutions extracted from *L. crispatus* CIP104459 exposed to 17β-estradiol 10^{-6}, 10^{-8} and 10^{-10} M was observed. However, the surface tension difference measured between solutions from control and 17β-estradiol-treated bacteria was only significant with estradiol 10^{-6} M (**Fig. 4C**).

17β-estradiol increases *L. crispatus* CIP104459 adhesion to vaginal mucosa epithelial cells *in vitro* but has limited effects on its biofilm formation activity. Finally, we investigated the effect of 17β-estradiol on adhesion and biofilm formation. Adhesion of *L. crispatus* CIP104459 to vaginal mucosa epithelial cells was assessed using the human vaginal VK2/E6E7 cell line. When bacteria were grown in the presence of 17β-estradiol at 10^{-8} M, a significant increase of adhesion to VK2/E6E7 cells was observed (+ 10.2 ± 3.8 %) (**Fig. 5**). No effect was observed with 17β-estradiol at 10^{-10} M, but also unexpectedly when bacteria were exposed to 17β-estradiol at 10^{-6} M. Measurement of the biofilm formation of *L. crispatus* CIP104459 by the crystal violet staining assay showed that 17β-estradiol at 10^{-6} and 10^{-8} M, but not 10^{-10} M, induced a limited but significant biofilm increase (**Fig. 6A**). To get further insights into the effects of 17β-estradiol on biofilm architecture, the 3D structure of *L. crispatus* CIP104459 biofilm was visualized by confocal laser scanning microscopy. However, bacteria grown in MRS were unable to adhere on the glass bottom of the microtiter plate and no biofilm could be observed in such conditions (*data not shown*). Therefore, bacteria were grown in a specific medium designed as “simulating genital tract secretion” (SGTS) medium[33] (**Table 1**). In this condition, we observed that 17β-estradiol had no visible effect on biofilm structure (**Fig. 6B**). Biofilm images analysis using the COMSTAT2 software revealed that the average thickness (µm), mean biomass volume ($\mu m^3/\mu m^2$) and roughness coefficient of control and 17β-estradiol-treated bacteria remained within the same values (**Fig. 6C**)

Bioinformatic study of the potential *L. crispatus* CIP104459 17β-estradiol receptor. As shown in this study, 17β-estradiol displayed effects on the physiology of *L. crispatus* CIP104459 at concentrations as low as 10^{-8} or 10^{-10} M for some phenotypes, suggesting the existence of a putative sensor for estradiol in this bacterium. The potential 17β-estradiol binding site was investigated using a bioinformatic approach based on the recently published draft genome of *L. crispatus* CIP104459[28] that was compared to the recently annotated genome of *L. crispatus* CO3MRSI1[34]. The putative translated ORF were then compared to the published sequence of nine eukaryotic 17β-estradiol binding proteins that were previously identified in the literature (**Table 2**). Two of these proteins, namely the estrogen-related receptor gamma (ERR3, UniProtKB - P62508) and the prohibitin-2 (PHB2, UniProtKB - Q99623) showed significant identity (33 and 24 %, respectively and similarity (55 and 46 %, respectively) with a *L. crispatus* CIP104459 protein designated as SPFH domain containing protein (NCBI Reference Sequence: WP_013086692.1). On the basis of the structure of the binding site in eukaryotic proteins, the potential association of 17β-estradiol to the *L. crispatus* SPFH domain containing protein was studied using AutoDock 4.2[35]. This 293 amino acids protein contains two domains, an N-terminal domain between amino acids 1 to 40 corresponding to a short extracellular sequence (amino acids 1 to 9) and a transmembrane helix (amino acids 10 to 25), and a long C-terminal sub-membrane domain organized as α helix and β-sheet. A 17β-estradiol potential binding site was identified in the sub-membrane domain in a region before α helix and β-sheet folds (**Fig. 7A**). Major amino acids involved in interaction between 17β-estradiol and the SPFH protein appear as glycine 45 and serine 94 (**Fig. 7B**). The generated binding value is particularly high (-7.8 Kcal/mol) and of statistical significance as it was identical in 53 % of calculated clusters.

Discussion

There is now ample evidence that the response of human commensal bacteria to peptidic hormones and neurohormones is essential to skin homeostasis[36]. Cutaneous bacteria such as *C. acnes* were also shown to express sensors for catecholamines (noradrenalin and adrenalin) suggesting that *C. acnes* acts as a relay between stress and acne[37]. Bacteria even produce small eukaryotic communication molecules such as histamine, glutamine and particularly γ-aminobutyric acid (GABA), which are well-known as eukaryotic communication molecules[36]. GABA is even considered as the universal inter-kingdom communication factor[38]. Conversely, sterols are rare in bacteria[39] and although bacteria develop in environments where the impact of steroid hormones is essential, the influence of steroids on bacterial physiology has been until yet poorly documented.

The absence of effect of 17β-estradiol on the growth of *L. crispatus* CIP104459 is consistent with previous studies showing that human neurohormones and neurotransmitters, including substance P, calcitonin gene related peptide (CGRP), natriuretic peptides, GABA and catecholamines, are generally without effect on the cultivability of commensal bacteria[36]. Conversely, it was demonstrated that steroid hormones, such as estradiol and androstenedione, have an inhibitory effect on the growth of *Helicobacter pylori*[40]. In this species, progesterone and other cholesterol derivatives even have a lethal activity that was attributed to membrane damaging[40-41]. However, these effects were only observed at high concentrations (> 50 µM)[40] and, at these non-physiological doses, many eukaryotic hormones including peptides previously mentioned can also exert non-specific anti-microbial activities[20]. Moreover, this antimicrobial activity of steroids is species dependent, as in the Gram-negative spirochete *Borrelia burgdorferi*, sterols have a limited effect on the viability and affect membrane permeability[42]. In fact, *Helicobacter* and *Borrelia* are representative of a small group of bacteria having significant amount of cholesterol in their membrane, whereas *Lactobacillus crispatus* is a Gram-positive Firmicute whose membrane contains minor amounts of sterol[39].

We examined the effect of 17β-estradiol on *L. crispatus* membrane fluidity by measurement of membrane anisotropy. A decreased of membrane anisotropy was observed after a continuous bacterial exposure for 18 h to 17β-estradiol, indicating a higher fluidity[29] which reveals membrane homoeostasis alteration. This phenotype could result either from a direct integration of the steroid into the membrane or from an adaptive response of the bacteria through hormone signal transduction. Numerous bacteria use the sterols-resembling hopanoids to regulate their membrane fluidity in response to environment conditions variations as temperature shifts[43-44], suggesting that 17β-estradiol may be able to insert into *L. crispatus* membranes. To get further insights into this mechanism, we next assayed membrane fluidity in a short-term exposure to 17β-estradiol. Minor differences of fluorescence anisotropy index were measured in bacteria grown to their mid-log-phase (6 h of growth). Remarkably, this phenomenon was attenuated when bacteria were grown to the entry of the stationary phase prior 17β-estradiol exposure (12 h of growth), and fully disappeared in case of late stationary phase grown bacteria. Thus, our data suggest that the increased membrane fluidity resulting from a continuous bacterial exposure for 18 h to 17β-

estradiol may not be the result of steroid insertion into the membrane, but rather a consequence of an adaptive response of the bacteria through hormone signal transduction. This hypothesis is also supported by the observation that the response to 17β-estradiol was preserved at 10^{-10} M, *i.e.* at a concentration where the number of steroid molecules accessible per bacterium should be very limited and not sufficient for leading to direct physico-chemical effects.

The surface polarity of *L. crispatus* exposed to 17β-estradiol was studied by the MATS technic. Changes were minor and only significant with one solvent (ethyl acetate) and estradiol 10^{-8} M. In parallel, the Lewis acid/base properties of the bacterial surface evolved from acido-basic to basic indicating that the cell surface polarity was poorly affected by continuous 17β-estradiol treatment. In addition, flow cytometry studies showed that the surface heterogeneity (granularity) of the bacteria exposed to 17β-estradiol remained similar to the control condition. Conversely, the mean size of the particles detected by flow cytometry analysis tended to decrease. The difference was not significant, probably as the signal combines two informations, the aggregation degree of the bacteria and their mean size. Indeed, all *Lactobacilli* are able to aggregate and *L. crispatus* is one of the species with the highest potential[45]. However, observation of *L. crispatus* by SEM revealed that 17β-estradiol did induced a significant decrease of the mean size of bacteria suggesting that the diminish of particle size observed in flow cytometry was not due to a difference of aggregation potential but likely to the lower size of *L. crispatus* cells. To support this hypothesis, it is interesting to note that the aggregation potential of *Lactobacilli* depends of outer surface S-layer proteins[46] and in the present study we observed that the physico-chemical properties of the bacterial surface were poorly affected by 17β-estradiol. Nonetheless, this study reveals that the response of *L. crispatus* to17β-estradiol involves a complex process leading to modification of the bacterial size.

L. crispatus CIP104459 colonies exposed to 17β-estradiol exhibit flattening and leaking phenotypes on Petri dishes. These observations suggested an increase of biosurfactant production. Biosurfactants are surface-active molecules reducing the air/water surface tension to low levels, but can also insert at any interface, abiotic or not. Thence some of them can insert into bacterial membranes and modify membrane fluidity[32], according to our data. The decrease of surface tension was dose related but was only significant in the presence of estradiol 10^{-6} M. Many *Lactobacilli* are known to produce molecules with biosurfactant activity, including glycoproteins such as surlactin[47]. Production of biosurfactant by vaginal *L. crispatus* was recently demonstrated[48]. The molecule appears as a complex non-homogenous lipopeptide since sophisticated chemical techniques, including Fourier transformed infrared spectroscopy and electron spray mass spectrometry were not sufficient to determine its complete structure[48]. This *L. crispatus* biosurfactant has interesting properties, including low cytotoxicity, stimulation of mucins mucoadhesion and even antagonism against *Candida spp.* adhesion[48]. In this regard, the effect of 17β-estradiol on *L. crispatus* biosurfactant production is of interest since this species is one of the major species of the vaginal microbiota[8-9] and this could have direct consequences in the preservation of vaginal homeostasis. This assumption is supported by the observation that 17β-estradiol 10^{-8} M increased the adhesion of *L. crispatus* CIP104459 to VK2/E6E7 vaginal cells. Unexpectedly, higher or

lower concentrations of 17β-estradiol had no significant effect. However, this might be explained by the behavior of biosurfactants at different concentrations. Indeed, the surfactive behaviour related to their amphiphilic character varies with their concentration just to a limit, designated as critical micellar concentration, while the biosurfactants saturate any interface and after they form micelles modifying the interactions with the environment[49]. Then, biosurfactants require to be present at an optimal concentration to exert their activity and 10^{-8} M should be in the range where 17β-estradiol is able to stimulates *L. crispatus* biosurfactant production at the optimal level.

The biofilm formation activity is also depending on bacterial adhesive properties, whilst other parameters including the secretory activity and matrix composition also have essential roles. In the present study, we observed using the crystal violet staining assay that 17β-estradiol was inducing a limited (<20%), but generally significant, increase of biofilm formation. Crystal violet is not specific and labels both bacterial bodies and matrix. Then, to characterize more precise the effect of 17β-estradiol, the structure of *L. crispatus* biofilm was studied by confocal microscopy. No difference of biofilm structure and mean thickness was observed. For crystal violet studies bacteria were grown in MRS medium and biofilm formation was studied in polystyrene tubes whereas for confocal microscopy, bacteria had to be grown in another medium (SGTS) and the biofilm was formed on glass surface. These technical differences could explain the absence of effect of 17β-estradiol on biofilm formation in confocal microscopy. Nevertheless, the impact of estradiol on biofilm formation appears marginal.

In order to identify the potential 17β-estradiol binding protein in *L. crispatus* CIP104459, we used a bioinformatic approach based on its recently published draft genome[28]. For reference, we selected a series of nine molecules identified as estradiol binding proteins in eukaryotes. Two of these proteins, the estrogen-related receptor gamma (ERR3) and prohibitin-2 (PHB2), showed significant homology with a *L. crispatus* CIP104459 protein designated as SPFH domain containing protein. SPFH are the initials of stomatin, prohibitin, flotillin and HflK/C, a familly of proteins associated to membrane lipid rafts[50]. The calculated binding value between 17β-estradiol and the SPFH domain containing protein was particularly high (-7.8 Kcal/mol), indicating a strong probability of interaction. The presence of the 17β-estradiol binding site in the sub-membrane region of the SPFH protein is coherent with the ability of 17β-estradiol to cross freely the membranes because of its amphiphilic structure. It is interesting to note that experimental studies performed in the Gram-negative bacterium *B. burgdorferi* also suggested an association of sterols with lipid rafts[39]. Moreover, it has been shown that these membrane microdomains should have more than structural functions in bacteria, one of them lying on signal transduction[51]. Therefore, collectively our data lay the foundation of a speculative but putative association of 17β-estradiol with SPFH domain containing protein in *L. crispatus* membrane lipid rafts that would be the first step in the induction of the bacterial response.

Taken together, this study reveals that 17β-estradiol can exert direct effects on *L. crispatus* by modifying its morphology and inducing biosurfactant production. This process might be of importance in the vaginal environment physiology by promoting adhesion of *L. crispatus* to the mucosa. This

phenomenon could also explain why *L. crispatus* administered to post-menopausal women, *i.e.* when the physiological production of estradiol is low, can hardly implant and exert their protective function[17]. These results should lead to the developments of new strategies for preserving or restoring vaginal homeostasis.

Methods

Bacterial stain and culture conditions. *Lactobacillus crispatus* CIP104459 was obtained from the Institut Pasteur Collection (CRBIP-Microorganism biobank catalogue, Paris, France). It was isolated in 1955 from a non-menopausal woman vaginal swab in the “La Croix-Rousse” maternity center (Lyon, France). In our laboratory, this strain was stored at -140°C in a cryofreezer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The draft genome sequence of *L. crispatus* CIP 104459 was recently determined and deposited in the DDBJ/ENA/GenBank under accession number VOMA00000000[28]. This bacterium was grown anaerobically in De Man, Rogosa and Sharpe (MRS) medium (VWR, Fontenay-sous-Bois, France) at 37°C under static conditions into conical 15 ml tubes (Corning®, Thermo Fisher Scientific, Waltham, Massachusetts, USA) filled with medium to maximal capacity. Culture stocks in glycerol / MRS 30% (v/v) were made and stored at −80°C before use. Pre-cultures were prepared anaerobically in MRS at 37°C for 48 h to reach the stationary growth phase. The density of the bacterial suspensions was determined by absorbance at 600 nm using a spectrophotometer (ThermoSpectronics, Cambridge, UK). Absence of contamination was controlled by plating onto MRS agar Petri dishes (VWR, Fontenay-sous-Bois, France). Growth of *L. crispatus* CIP104459 was monitored over 48 h

For monitoring of the growth kinetic, bacteria were layered in microplates, at an initial OD_{600nm} = 0.08 and incubated in anoxic conditions over 48 h at 37°C with shaking (360 rpm) for ten seconds before each measure point using a multimode microplate reader (Tecan Group Ltd., Männedorf, Switzerland). Growth curves were obtained by automatic measurement every 30 min. Generation time and lag phase were calculated from the software of the microplate reader.

Tested molecule. 17β-estradiol (Sigma-Aldrich, Saint-Quentin-Fallavier, France) being poorly soluble in aqueous media, a stock solution was made by dilution in ethanol 100%. For bacterial treatment, this solution was dissolved in MRS so that the final concentration of ethanol in MRS was always kept at 0.1% v/v. Controls were performed using the same percentage of ethanol in MRS. Preliminary studies allowed to verify the absence of effect of ethanol 0.1% on *L. crispatus* CIP104459.

Evaluation of bacterial membrane fluidity. *L. crispatus* CIP104459 was grown in the absence or presence of 17β-estradiol over 18 h from the onset to the end of the experiment. After incubation, bacteria were collected by centrifugation (7,500 ×*g*, 10 min) and washed two times at room temperature in 10 mM $MgSO_4$. The pellets were resuspended in the same solution and the OD_{600nm} was adjusted to 0.1. In each 1mL aliquot of the bacterial suspension, 1 µL of 1,6-diphenyl-1,3,5-hexatriene (DPH) 4 mM in tetrahydrofuran (Sigma-Aldrich, Saint-Quentin-Fallavier, France) was added. Aliquots were then incubated in the dark for 30 min at 37 °C to allow incorporation of the probe into the bacterial membrane.

Fluorescence polarization was measured using a Spark 20 M multimode microplate reader, equipped with an active temperature regulation system (Te-CoolTM, Tecan Group Ltd., Männedorf, Switzerland). Excitation and emission wavelengths were set at 365 and 425 nm, respectively. Each measure was realized in triplicate. Membrane anisotropy (r) was calculated according to Lakowicz[52]. Data were analyzed using the SparkControlTM software 2.1 (Tecan Group Ltd., Männedorf, Switzerland). The relation between fluorescence polarization and membrane fluidity is inverse. When anisotropy values increase that is corresponding to a decrease of membrane fluidity and vice versa. In a second series of experiments bacteria were grown for 6, 18 or 24 h in normal MRS medium collected and rinsed as previously describes and exposed to 17β-estradiol after incorporation of the fluorescent probe. Evolution of fluorescence polarization was measured over 3 h using the same equipment.

Determination of bacterial surface polarity. The surface polarity and Lewis acid-base properties of *L. cristapus* CIP104459 exposed or not to 17β-estradiol were studied using the microbial adhesion to solvents (MATS) technic[53]. Bacteria were grown in MRS and harvested by centrifugation (7,500 *×g*, 10 min) after 18 h of incubation. The pellets were washed twice in phosphate buffer saline (PBS) (Lonza™, Thermo Fisher Scientific, Waltham, Massachusetts, USA) to remove traces of culture medium. Two solvent couples: chloroform/hexadecane and ethyl acetate/n-decane were employed. For each condition, 1.2 mL of bacterial suspension at OD_{400nm} = 0.8 was mixed for 60 s with 0.2 mL of each solvent. After incubation for 15 min and separation of the aqueous and organic phases, the OD_{400nm} of the aqueous phase was measured. The percentage of bacteria accumulating in each organic compartment (solvent phase) was calculated using the following equation with [AO] = OD_{400nm} of the aqueous phase without solvent and [A] = OD_{400nm} of the aqueous phase after exposure to the solvent:

$$\% \text{ solvent affinity} = (1-A) / A0 \times 100$$

All experiments were conducted in triplicate or more.

Investigation of bacterial aggregation and morphology. Formation of aggregates by *L. crispatus* CIP104459 was studied by the sedimentation technique described by Vandevoorde *et al.*[30]. Briefly, cultures of *L. crispatus* CIP104459 grown in MRS for 18 h under anoxic static conditions at 37°C and in the absence or presence of 17β-estradiol, were harvested by centrifugation (7,500 *xg*, 10 min, room temperature), washed twice in PBS, and resuspended in 10 mL of the same medium. After 1.5 min of agitation (vortex) which defined the T=0 of the experiment, the variation of OD_{600nm} of the suspension was monitored over 30 min using a spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The percentage of auto-aggregation after 30 min was calculated by the following equation:

$$\% \text{ auto-aggregation} = ([(OD0min - OD30min)/OD0min])/0D0min \times 100$$

Where OD0min is the initial OD_{600nm} at T = 0 and OD30min is the final OD_{600nm} after 30 min.

The potential auto-aggregation and structure of *L. crispatus* CIP104459 was also studied by flow cytometry using a CytoFlex S flow cytometer (Beckman coulter Life science, Indianapolis, USA) and the CytExpert software. After 18 h of culture in the absence or presence of 17β-estradiol, the bacterial suspension was harvested by centrifugation as previously described and resuspended in PBS. Bacteria were not stained and aliquots (200 µL) were distributed in 96 wells microplates (Thermo Fisher Scientific, Waltham, Massachusetts, USA). After incubation in static condition for 30 min the samples were submitted to flow cytometry analyses. A minimum of 10,000 events at $OD_{488 \pm 4\ nm}$ (SSC channel) and $OD_{525 \pm 20\ nm}$ (FSC channel) were recorded at a flow rate of 10 µL.min^{-1} in each condition. Data were analyzed using the Cytexpert software. The percentage of the population organized as aggregates was corresponding to the fraction of events appearing in the Q1-UR quarter of the graph (red zone). The size is given by the FSC-A (horizontal) axis and the percentage of small bacteria appeared in the Q1-UL and Q1-LL zones. The surface granulometry is plotted in the SSC-A (vertical) axis. The increase of complexity appears in the Q1-UL and Q1-UR areas of the graph. Measures were realized in triplicate.

The precise morphology of *L. crispatus* CIP104459 was studied by scanning electron microscopy using a TENEO VolumeScope microscope (FEI, Hillsboro, OR, USA) under 10 kV. For that, *L. crispatus* grown for 18 h in the absence (control) or presence of 17β-estradiol were collected by centrifugation (7,500 x*g*, 10 min). For fixation, bacterial pellets were immersed in 1 mL 2.5% glutaraldehyde in 1 M phosphate buffer, pH 7.1 for 1 h. Sample preparation was carried out as previously described[54]. After HMDS treatment (hexamethyldisilazane), the surface of the filter was coated with an electrically conductive 25 nm thick layer of platinum alloy coating in a sputter-coater vacuum chamber (LEICA EM ACE600 sputter coater, Wetzlar, Germany).

Evaluation of biosurfactant production. Cultures on MRS Petri dishes showed that *L. crispatus* CIP104459 grown in the presence of 17β-estradiol were spreading and flowing on the surface of the culture medium suggesting biosurfactant production. Then, MRS Petri dishes were inoculated in surface by spreading 100 µL of control or 17β-estradiol treated bacterial suspensions to obtain a continuous bacterial mat. As described by Meylheuc *et al.*[55], after 24 h of anaerobic culture at 37°C, the bacterial mat was gently scraped and resuspended in 15 mL Volvic water (selected for its neutral effect on surface tension). Then, the suspension was shaken by vortex for 3 min and centrifuged two times for 30 min at 4°C (10,000 *xg*) to remove all bacteria and fragments. The supernatant was collected and stored at 4°C.

The presence of biosurfactant in solution was first investigated by the sessile drop technique[56] by deposit on a polystyrene surface of 20 µL drops of supernatant and visualization of the contact angle with the surface. Measurement of this contact angle can be used to calculate the surface tension between the solution and the surface. However, this value being influenced by the properties of the surface, another approach, the pendant drop method was preferred. The shape of the drops of supernatant was analyzed using a drop shape analyser DSA30 temperature controlled tensiometer equipped with a video camera (Kruss, Hamburg, Germany). The surface tension, or interfacial tension, was calculated using the tensiometer drop shape analysis software based on the Young-Laplace

equation[57]. In order to take in consideration any potential variation due to the bacterial biomass collected, calculated surface tension values were correlated to the OD_{600nm} of the bacterial suspension measured immediately after scrapping of the mat on the Petri dishes.

Measure of bacterial adhesion to vaginal cells. Adhesion of *L. crispatus* CIP104459 to vaginal cells was studied *in vitro* using the VK2/E6E7 cells line (ATCC CRL-2616). This vaginal cell line was developed from the vaginal mucosal tissue of a healthy pre-menopausal women. It was maintained and propagated using keratinocyte serum-free medium (KSFM) (Thermo Fisher Scientific, Waltham, Massachusetts, USA), supplemented with 0.05 mg/mL bovine pituitary extract, 0.1 ng/mL human recombinant EGF and additional calcium chloride 44.1 mg/mL as recommended by the provider. Cells were replicated into fresh medium every 3 days after reaching confluence. For evaluation of bacterial adhesion, VK2/E6E7 cells were exposed to *L. crispatus* CIP104459 grown for 18h in MRS medium with or without 17β –estradiol at an MOI of 100 bacteria /cell. Experiments were performed using KSFM without antibiotics and VK2/E6E7 cells reaching a minimum of 80% confluence. After 1 h of bacterial interactions, the medium was removed slowly to withdraw planktonic bacteria and rinsed carefully with KSFM without antibiotics. Then VK2/E6E7 cells were disrupted by addition of 0.1% triton X-100 in PBS. The lysate was diluted in MRS medium and plated on MRS agar Petri dishes. The number of cells adherent bacteria was deduced from direct counting of growing *L. crispatus* colonies after 48 h culture at 37°C in anoxic conditions.

Determination of biofilm formation activity and structure. Biofilm formation by *L. crispatus* CIP104459 was initially studied using the crystal violet technique according to a procedure adapted from O'Toole[58]. Bacteria grown in MRS for 48h were adjusted to OD_{600nm} = 0.1 in a final volume of 1 mL and distributed in sterile 24-well polystyrene plates (Falcon®, Durham, USA). Plates were incubated anaerobically at 37°C without agitation for 48 hours in a Whitley A85 Workstation. At the end of the incubation period, the medium was discarded by aspiration and non adhered bacteria were removed by washing twice with physiologic water (PW, NaCl 0.9%). Biofilms were stained by incubation with crystal violet (0.1% w/v in sterile pure 18.2MΩ water) for 10 minutes at room temperature. The excess of dye was removed by three washing steps in pure 18.2MΩ water. The crystal violet adsorbed in the biofilm matrix and on bacteria was dissolved with absolute ethanol and the OD_{595nm} of the solution was determined using an automated plate reader (Te-CoolTM, Tecan Group Ltd., Männedorf, Switzerland).

L. crispatus CIP104459 grown in MRS was unable to adhere and form biofilm on glass surfaces, as required for optical microscopy. A more complete medium, specific to microorganisms of the vaginal microflora, designated as "simulating genital tract secretion" (SGTS) medium was produced according to Geshnizgani and Onderdonk[33] (**Table 1**). Then, *L. crispatus* CIP104459 pre-culture were realized in MSR and bacteria were transferred in SGTS medium for biofilm studies. 17β-estradiol, or an equivalent amount of ethanol in water, was administered from the onset of the biofilm formation study. The absence of effect of 17β-estradiol on *L. crispatus* CIP104459 growth in SGTS medium was controlled in preliminary studies (**Suppl Fig. 3**). For confocal microscopy, bacteria were harvested by centrifugation (7,500 *xg*, 10 min) and re-suspended in SGTS medium at OD_{600nm} = 0.1. Suspension aliquots (1 mL) containing or not

17β-estradiol were layered in 24-well microplates with flat glass bottom (Sensoplate, Greiner Bio-One, Germany) and incubated for 48 h in static and anoxic conditions to allow biofilm formation. Wells were washed twice with PW to remove remaining planktonic bacteria and biofilms were stained with SYTO 9 Green Fluorescent Nucleic Acid Stain (Thermofisher, Waltham, Massachusetts, USA). Stained samples were examined under an LSM 710 inverted confocal laser scanning microscope (Zeiss, Marly-le-Roi, France) using the Zen 2009 software package (version 12.0.1.362). Images were reconstructed and analyzed using the COMSTAT2 software. Biofilm average thickness (µm), mean biomass volume (µm3/ µm2) and roughtness coefficient were calculated over a minimum of 30 observations in each condition. All experiments were repeated at least of three times.

Bioinformatic studies. As a steroid, 17β-estradiol is an amphiphilic molecule and is not showing reactive groups allowing to form directly covalent conjugates with dyes or tracers. In addition, because of its small size, minor chemical modifications can deeply affect its functions. Incorporation of isotopes remains the sole technique to investigate experimentally its binding to potential receptors, but these techniques are submitted to heavy regulatory constraints. Then we decided to use bioinformatics approaches to investigate potential 17β-estradiol binding site(s) in *L. crispatus*. As previously mentioned, the draft genome of *L. crispatus* CIP104459 was recently published[28]. This draft genome was aligned by BLASTp on ExPASy (https:// web.expasy.org/blast) with the annotated sequence of *L. crispatus* CO3MRSI1[34] to improve its definition. The FASTA amino-acid sequence of the *L. crispatus* CIP104459 genome was then aligned with the sequence of known human estrogens receptors listed in **Table 2**. A bacterial protein showing high homology with human estrogens receptors was identified and submitted to molecular docking *in silico* using 17β-estradiol as potential ligand. *L. crispatus* proteins 3D models were generated using RaptorX Structure[59] and visualized using Python Molecular Viewer V1.5.6. Prediction by alignment on the crystalized structure of the corresponding eukaryotic ortholog. The potential binding of 17β-estradiol to the identified sensor protein was studied using AutoDock 4.2[35]. Binding values were generated using the Lamarkian Genetic Algorithm of AutoDock 4.2. All calculations were performed using a DELL Precision T7610 computer equipped with four hard disks (4 To each, for a total of 12 To under RAID5).

Statistical analysis. Statistical significance of experimental values was evaluated using the Prism GraphPad online tool (https://www.graphpad.com/quickcalcs/ttest1/). Data were analyzed using unpaired (two sample) two-tailed *t* test to calculate *p* values. Mean with standard error of the mean (SEM) were calculated and plotted.

Declarations

Acknowledgements

The authors would like to thank the Cosmetic Valley world cluster and CNRS GDR CNRS 3711 Cosm'Actifs for their scientific and relational support. This work was supported by the FUI program Gynceutics (BPI, Région Normandie, Région Centre, Région Aquitaine and European Union - FEDER).

Authors' contributions

M.C., A.S., P-J.R., J.V., M.B. and O.M. contributed to the conception of the approach and conducted experiments. A.T., A.LT, M.K., G.R. and C D-P contributed to the analysis of the data. C.K, C.P., S.C. and M.G.J.F coordinated the study. M.C, S.C and M.G.J.F designed and wrote the manuscript.

Competing interests. The authors have the following interests: Agathe Le Tirant is member of Sequen's Lab, Madina Karsybayeva is managing Remedials Laboratory, Coralie Kremser belongs to GymoPharm, Gérard Redziniak is head of Cosmetic Inventions society. There no product or marketing interest to declare. This does not alter the authors' adherence to all policies on sharing data and materials, as detailed online in the guidelines for authors. Other authors have no conflict of interest to declare.

Ethics approval and consent to participate. Not applicable, this study did not involve clinical trial.

Consent for publication. All authors have read and approved the manuscript. LMSM accepts to be responsible for the publication fees.

Availability of data and material. All experimental raw data are available upon simple demand to the authors.

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Tables

Table 1. Composition of the simulating genital tract secretion (SGTS) medium

Component	*Final concentration (g/L)*
Part 1[a]	
NaCl	3.5
KCL	1.5
K2HPO4	1.74
KH2PO4	1.36
Dextrose	10.8
Cysteine HCL	0.5
Part 2[b]	
Glycogen	1
Mucin	0.25
Tween 20	0.2
Urea	0.5
Hemin	0.5
Albumine	0.2
*MgSO4**	0.3
*NaHCO3**	0.04
Part 3	
Vitamin mix[d]	5mL of 100X solution

*Solutions were sterilized by passage through a membrane filter (pore size, 0.22μm).

[a]Prepared by dissolving the components in pure 18.2MΩ water. After adjustment of pH to 7.2 with NaOH, the solution was autoclaved for 15 min at 121°C and then cooled to room temperature.

[b]Part II consisted of eight components prepared separately. Solutions 1 to 5 were autoclaved

for 15 min at 121°C.

Table 2. List and references of the nine eukaryotic 17β-estradiol binding proteins tested for their potential homology with *Lactobacillus crispatus* CIP104459 proteins.

UniProtKB Accession Number	Human gene	Name
P03372	ESR1	ESR1_HUMAN Estrogen receptor
O95718	ESRRB	ERR2_HUMAN Steroid hormone receptor ERR2
Q99527	GPER1	GPER1_HUMAN G-protein coupled estrogen receptor 1
P62508	ESRRG	ERR3_HUMAN Estrogen-related receptor gamma
Q92731	ESR2	ESR2_HUMAN Estrogen receptor beta
Q99623	PHB2	PHB2_HUMAN Prohibitin-2
Q8NI08	NCOA7	NCOA7_HUMAN Nuclear receptor coactivator 7
P49888	SULT1E1	ST1E1_HUMAN Estrogen sulfotransferase
Q86YN6	PPARGC1B	PRGC2_HUMAN Peroxisome proliferator-activated receptor gamma coactivator 1-beta

Figures

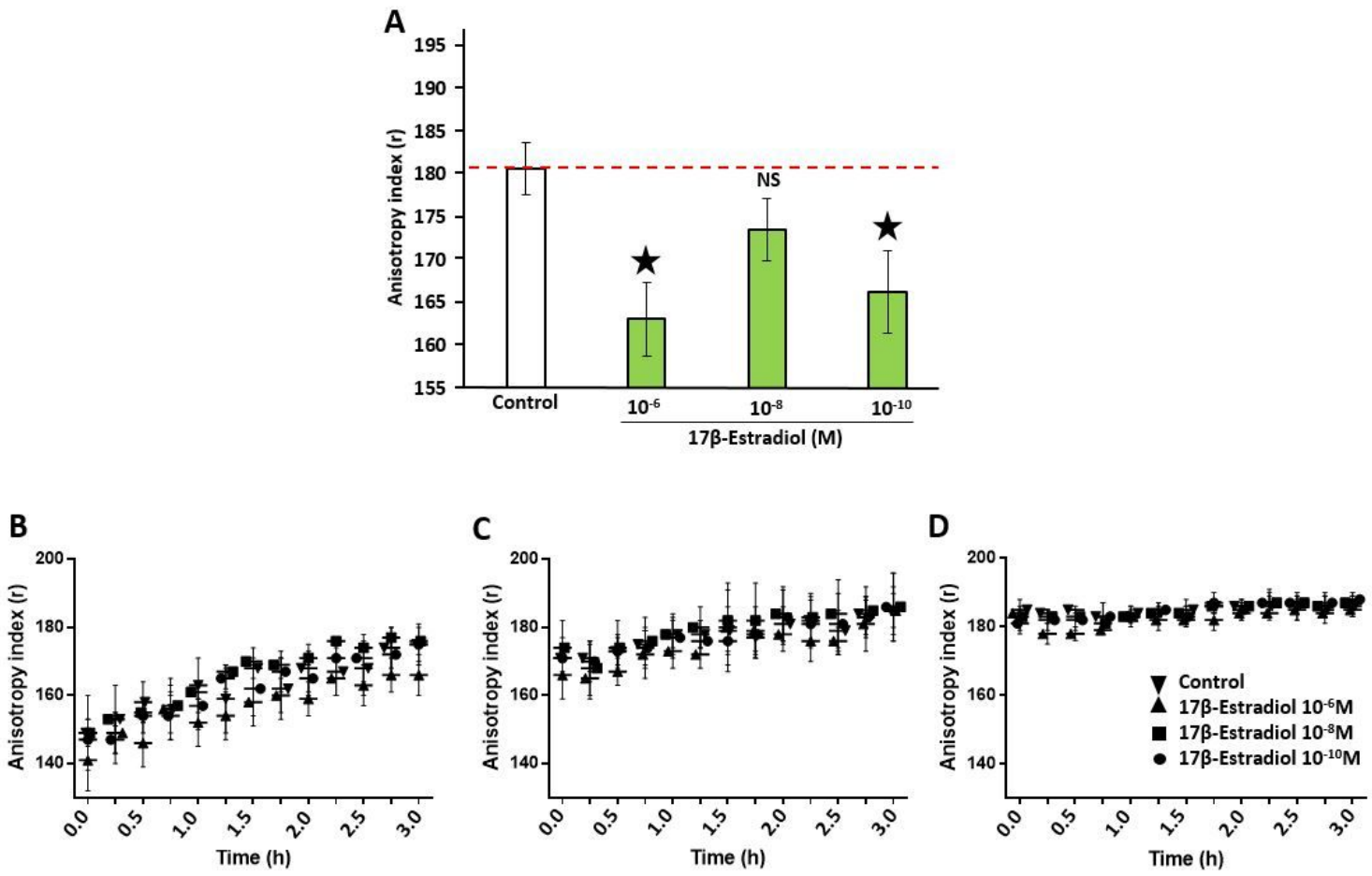
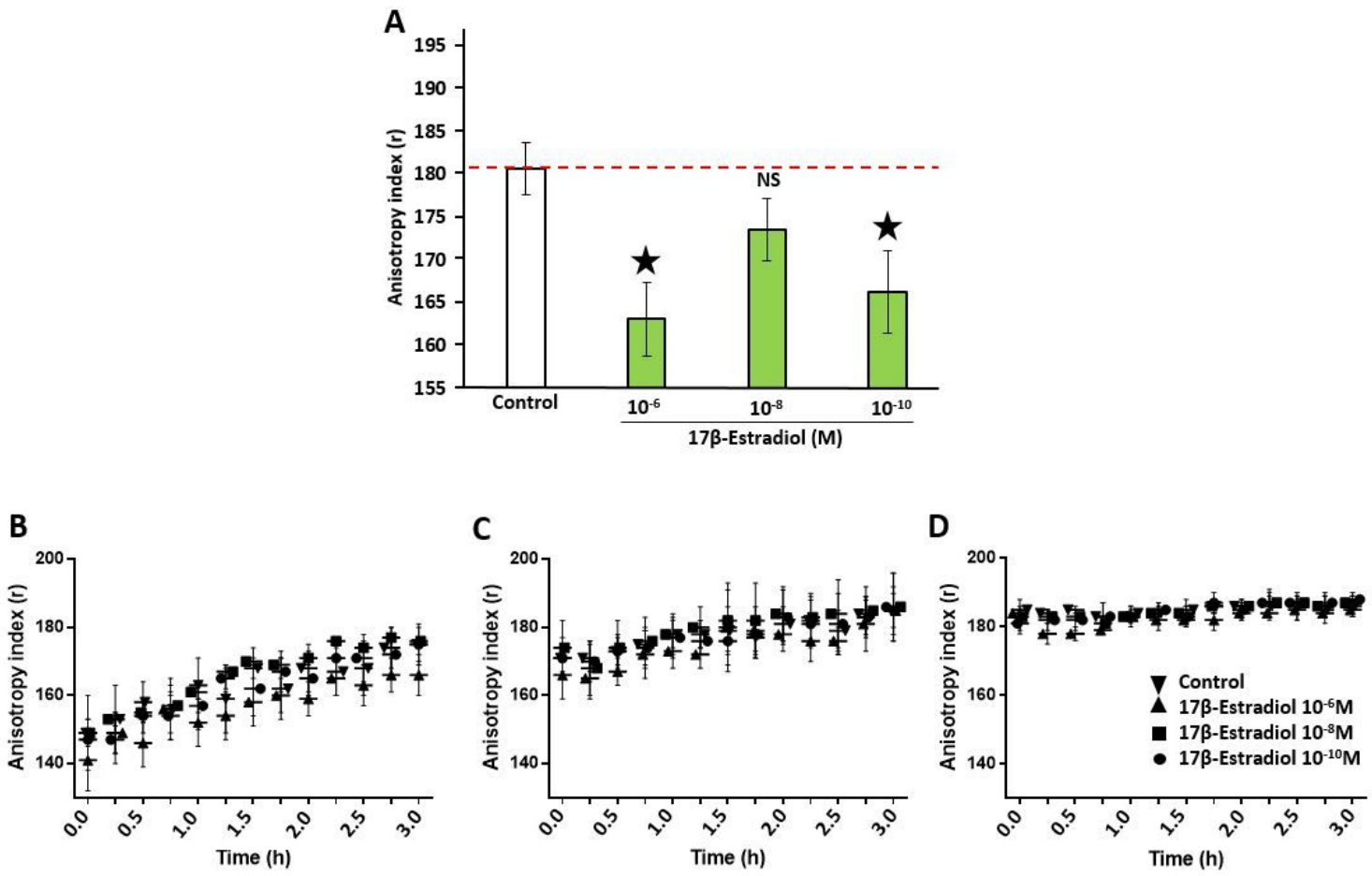


Figure 1

Effect of 17β-estradiol on Lactobacillus crispatus CIP104459 membrane fluidity. (A) Membrane fluidity (anisotropy index) of L. crispatus CIP104459 grown for 18 h in the absence (control) or presence of 17β-estradiol (10-6, 10-8 and 10-10 M). (B) Membrane fluidity (anisotropy index) of L. crispatus grown for 6 h in normal MRS and subsequently exposed to 17β-estradiol (10-6, 10-8 or 10-10 M). (C) Membrane fluidity (anisotropy index) of L. crispatus grown for 12 h in normal MRS and subsequently exposed to 17β-estradiol (10-6, 10-8 or 10-10 M). (D) Membrane fluidity (anisotropy index) of L. crispatus grown for 24 h in normal MRS and subsequently exposed to 17β-estradiol (10-6, 10-8 and 10-10 M). Values and curves are the means ± SEM of three independent studies. (NS: not significant; ★= $p < 0.05$).



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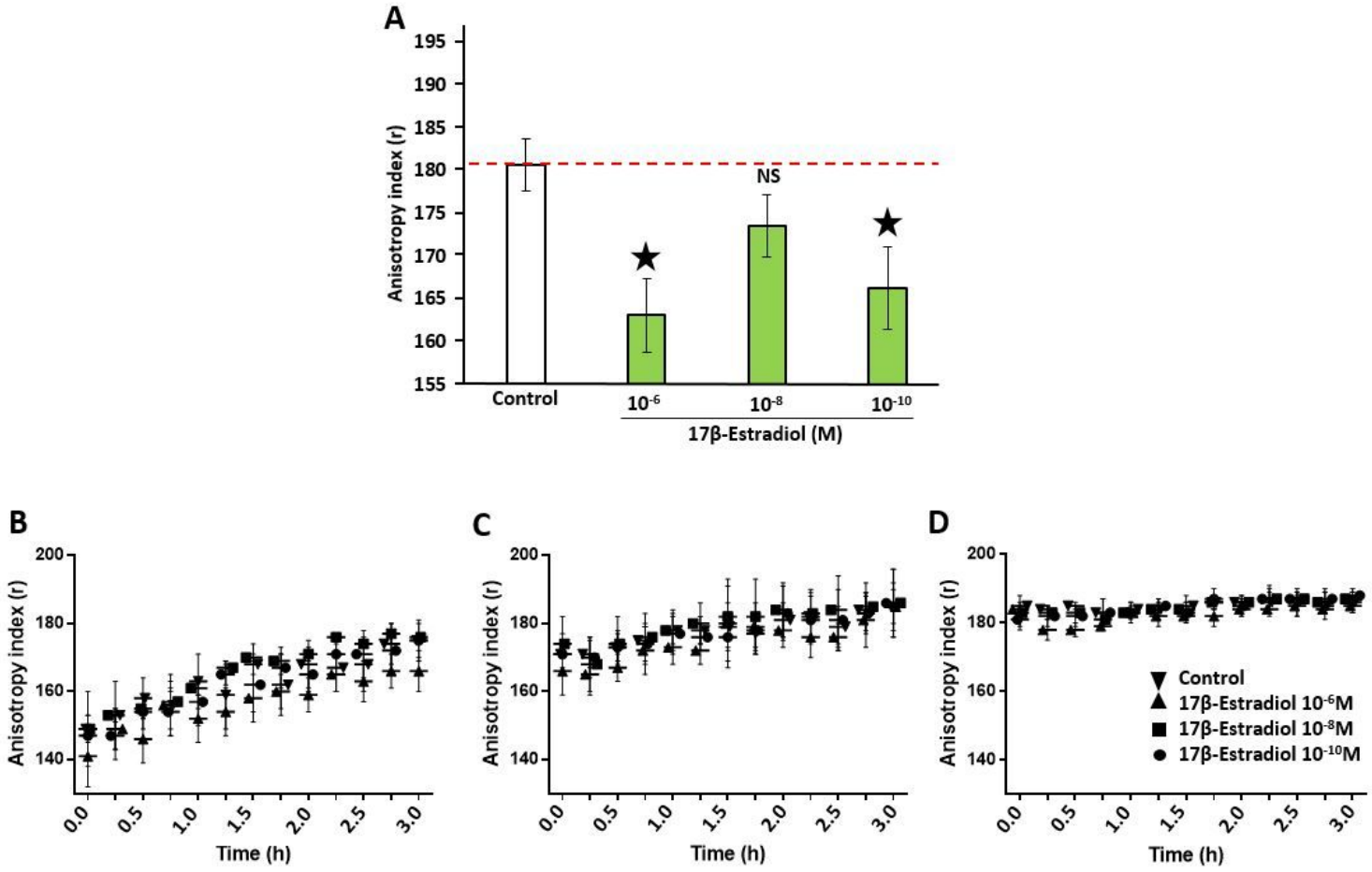
Effect of 17β-estradiol on Lactobacillus crispatus CIP104459 membrane fluidity. (A) Membrane fluidity (anisotropy index) of L. crispatus CIP104459 grown for 18 h in the absence (control) or presence of 17β-estradiol (10-6, 10-8 and 10-10 M). (B) Membrane fluidity (anisotropy index) of L. crispatus grown for 6 h in normal MRS and subsequently exposed to 17β-estradiol (10-6, 10-8 or 10-10 M). (C) Membrane fluidity (anisotropy index) of L. crispatus grown for 12 h in normal MRS and subsequently exposed to 17β-estradiol (10-6, 10-8 or 10-10 M). (D) Membrane fluidity (anisotropy index) of L. crispatus grown for 24 h in normal MRS and subsequently exposed to 17β-estradiol (10-6, 10-8 and 10-10 M). Values and curves are the means ± SEM of three independent studies. (NS: not significant; ★= $p < 0.05$).



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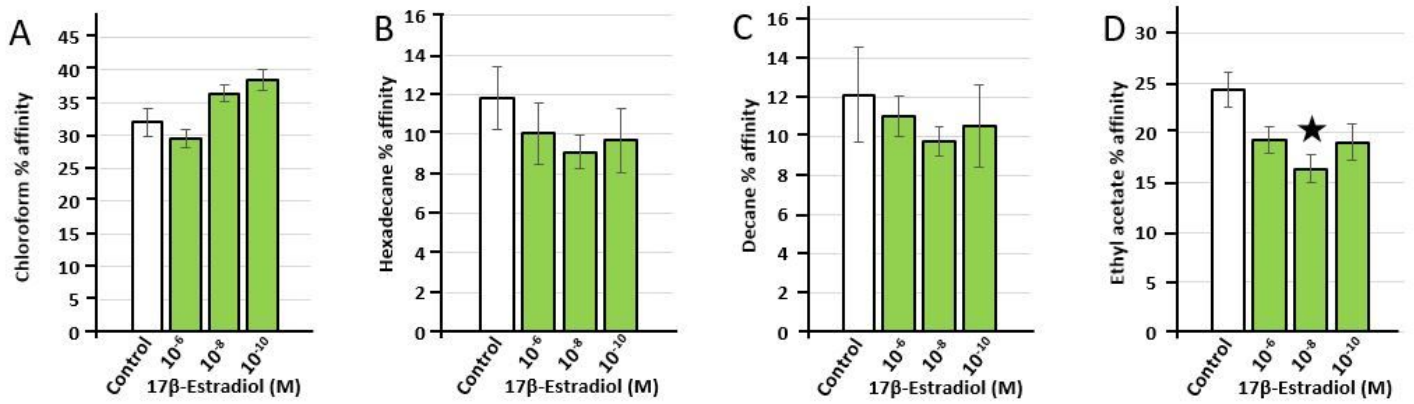
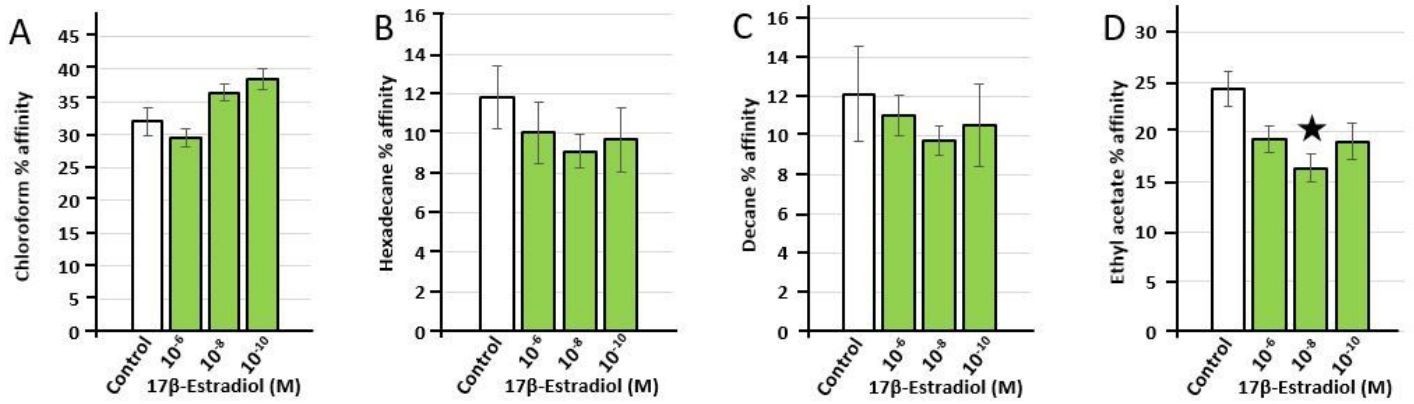


E

	Control	17β-Estradiol 10^{-6}M	17β-Estradiol 10^{-8}M	17β-Estradiol 10^{-10}M
$\Delta_{basicity}$ (HD-CH) ± SEM	20 ± 2.19	20 ± 1.58	27 ± 2.42	29 ± 1.82 ★
$\Delta_{acidity}$ (DE-AE) ± SEM	12 ± 1.62	8 ± 1.61	7 ± 2.09	9 ± 1.87
Lewis acid/base properties	acido-basic	basic	basic	basic

Figure 2

Effect of 17β-estradiol (10-6, 10-8 and 10-10 M) on the affinity to solvants and Lewis acid/base surface properties of Lactobacillus crispatus CIP104459. (A) Partition between water and chloroform. (B) Partition between water and hexadecane. (C) Partition between water and decane. (D) Partition between water and ethyl acetate. (E) Lewis acid/base behavior of L. crispatus CIP104459 with the two solvents couples hexadecane (HD)/chloroform (CH) and decane (DE)/ethyl acetate (EA). Values are the means ± SEM of three independent studies. (★= $p < 0.05$).



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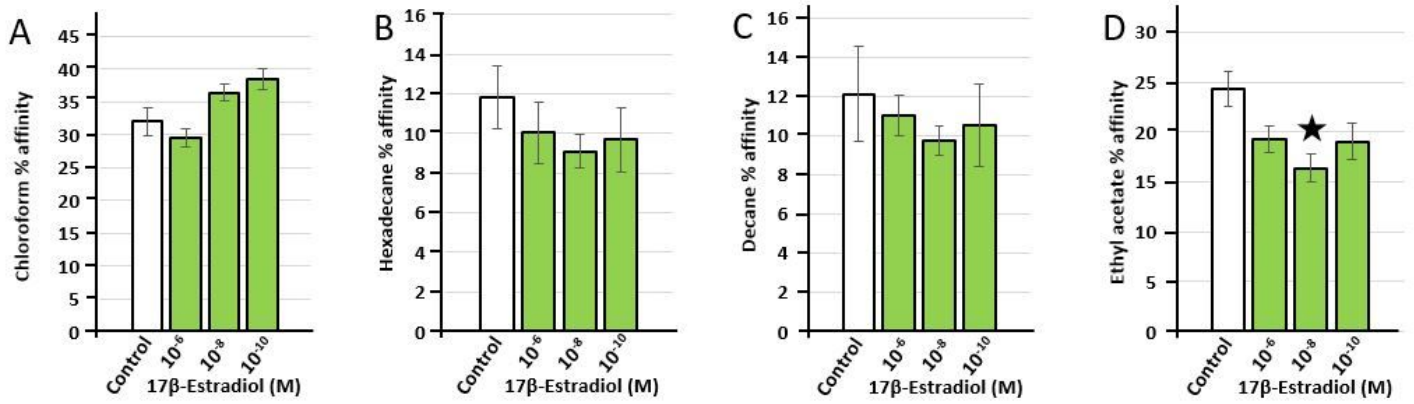

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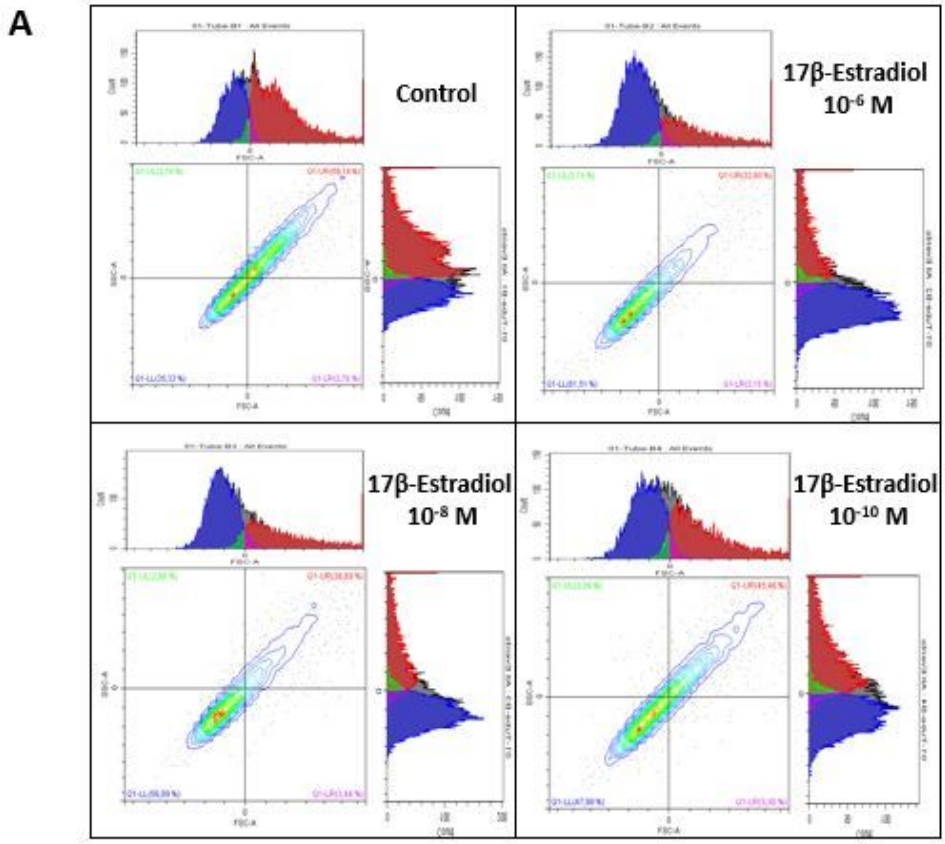
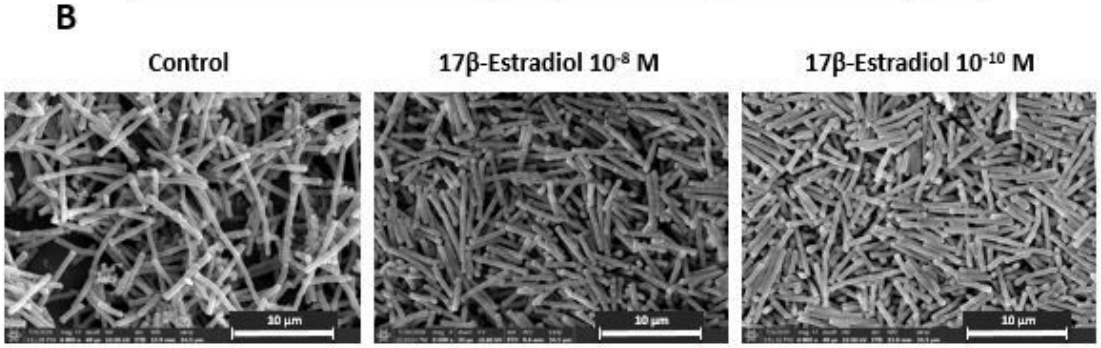




C

★★★

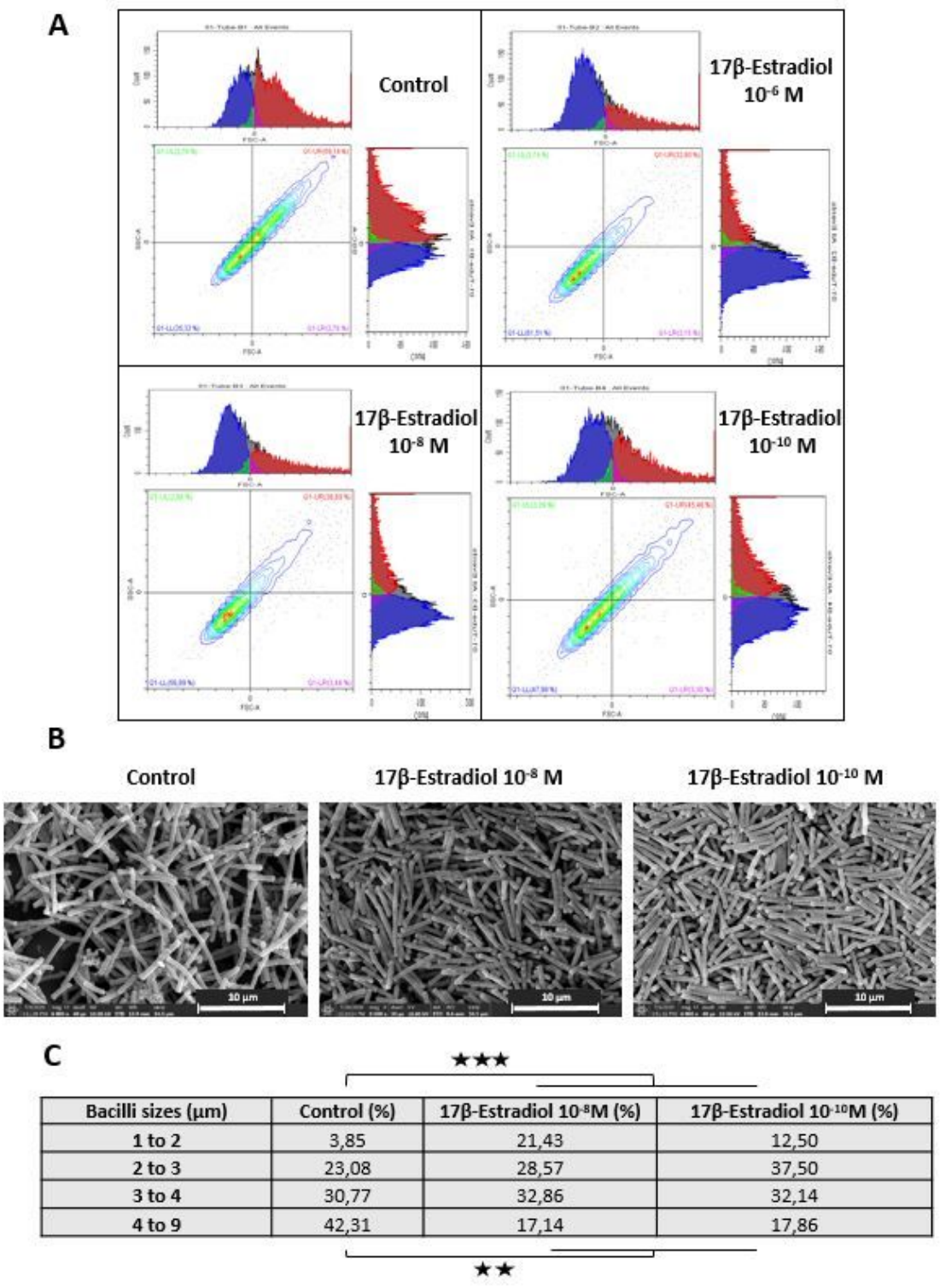
Bacilli sizes (µm)	Control (%)	17β-Estradiol 10-8M (%)	17β-Estradiol 10-10M (%)
1 to 2	3,85	21,43	12,50
2 to 3	23,08	28,57	37,50
3 to 4	30,77	32,86	32,14
4 to 9	42,31	17,14	17,86

★★

Figure 3

Effect of 17β-estradiol on the aggregation potential and morphology of Lactobacillus crispatus CIP104459. (A) Flow cytometry diagrams illustrating the effect of 17β-estradiol (10-6, 10-8 and 10-10 M) on the mean size and aggregation (X axis = forward scattering fraction (FSC)) and surface heterogeneity (Y axis = side scatter (SSC)) of the bacteria. (B) Scanning electron microscopy of control and 17β-estradiol treated bacteria. Scale bar = 5 µm. (C) Table showing the relative percentage of bacteria

classified by size after culture in the absence or presence of 17β-estradiol (10-8 or 10-10 M). (** = $p < 0.01$; *** = $p < 0.001$).



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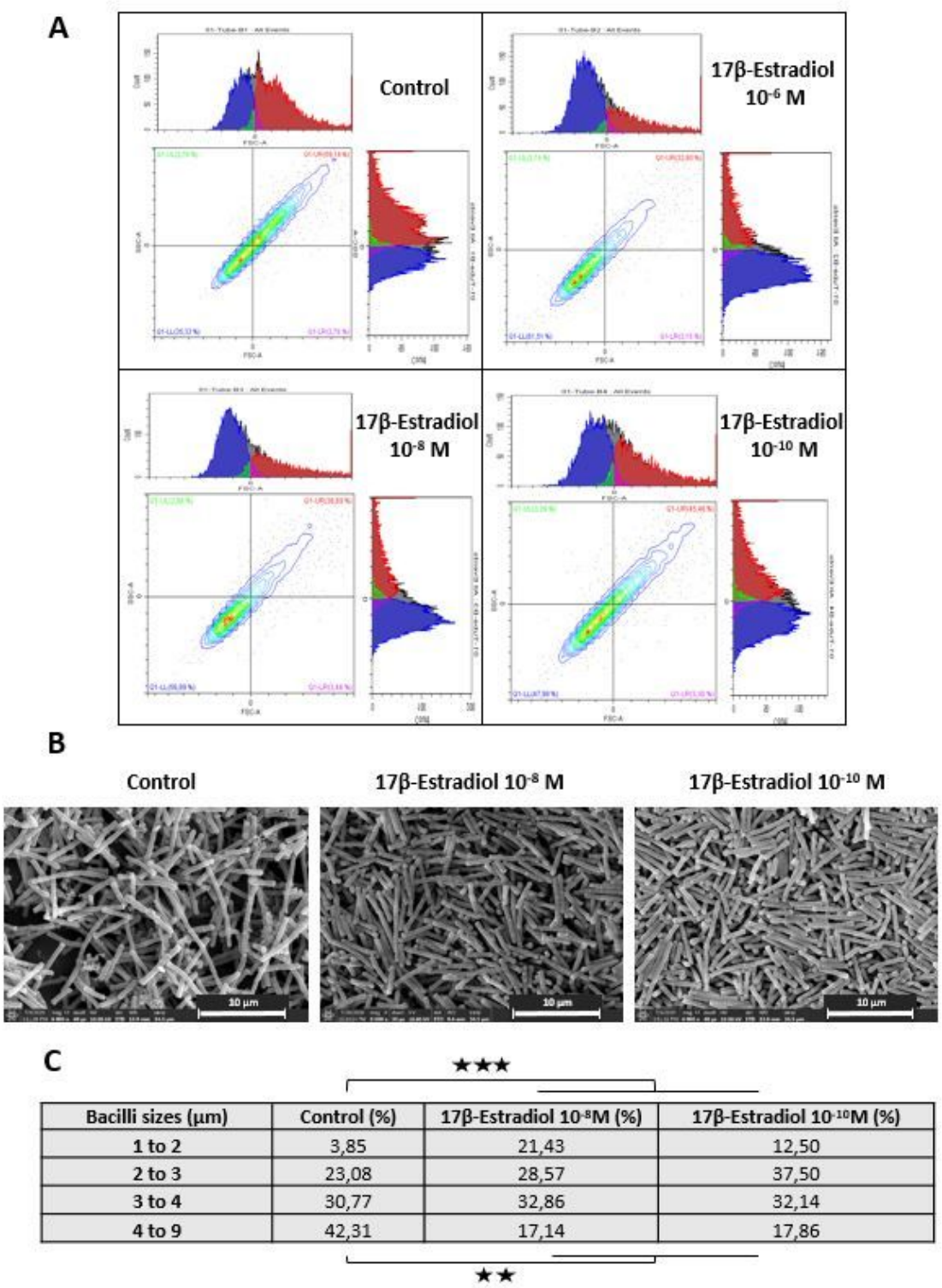

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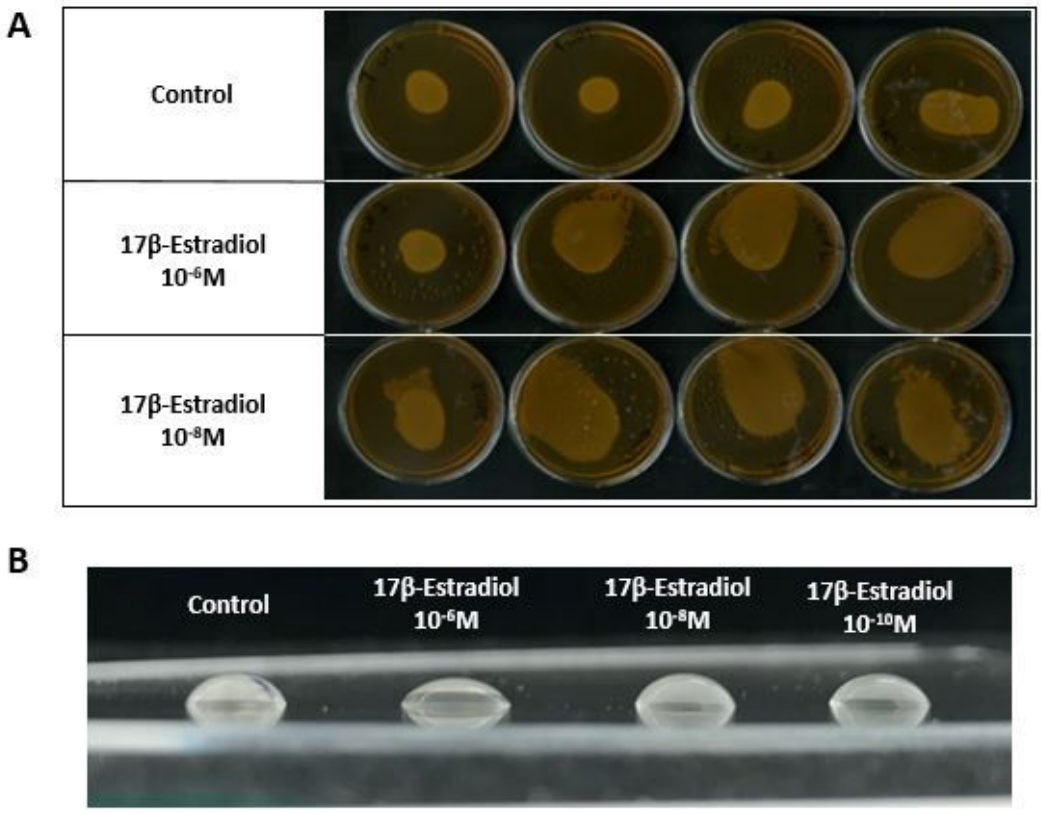
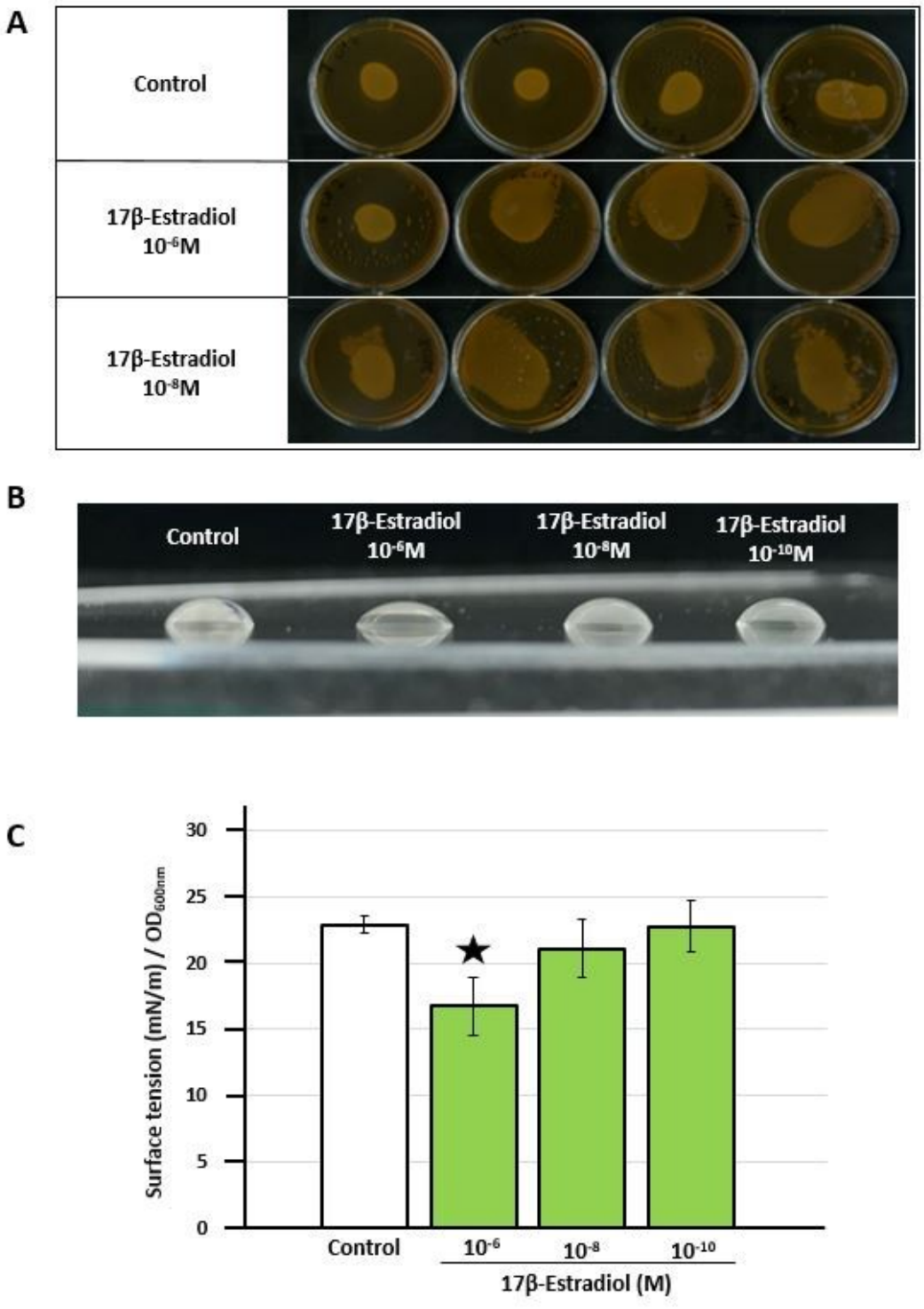


Figure 4

Effect of 17β-estradiol on Lactobacillus crispatus CIP104459 biosurfactant production. (A) Cultures of L. crispatus CIP104459 on MRS agar medium after exposure to 17β-estradiol (10-6 and 10-8 M). (B) Typical shape of drops of solution obtained after rinsing of bacterial mat grown on Petri dishes in the absence or presence of 17β-estradiol (10-6, 10-8 and 10-10 M). (C) Surface tension value of solutions extracted from L. crispatus CIP104459 exposed to17β-estradiol 10-6, 10-8 and 10-10 M measured by the pendant drop method. Values are the means ± SEM of three independent studies. (= $p < 0.05$).



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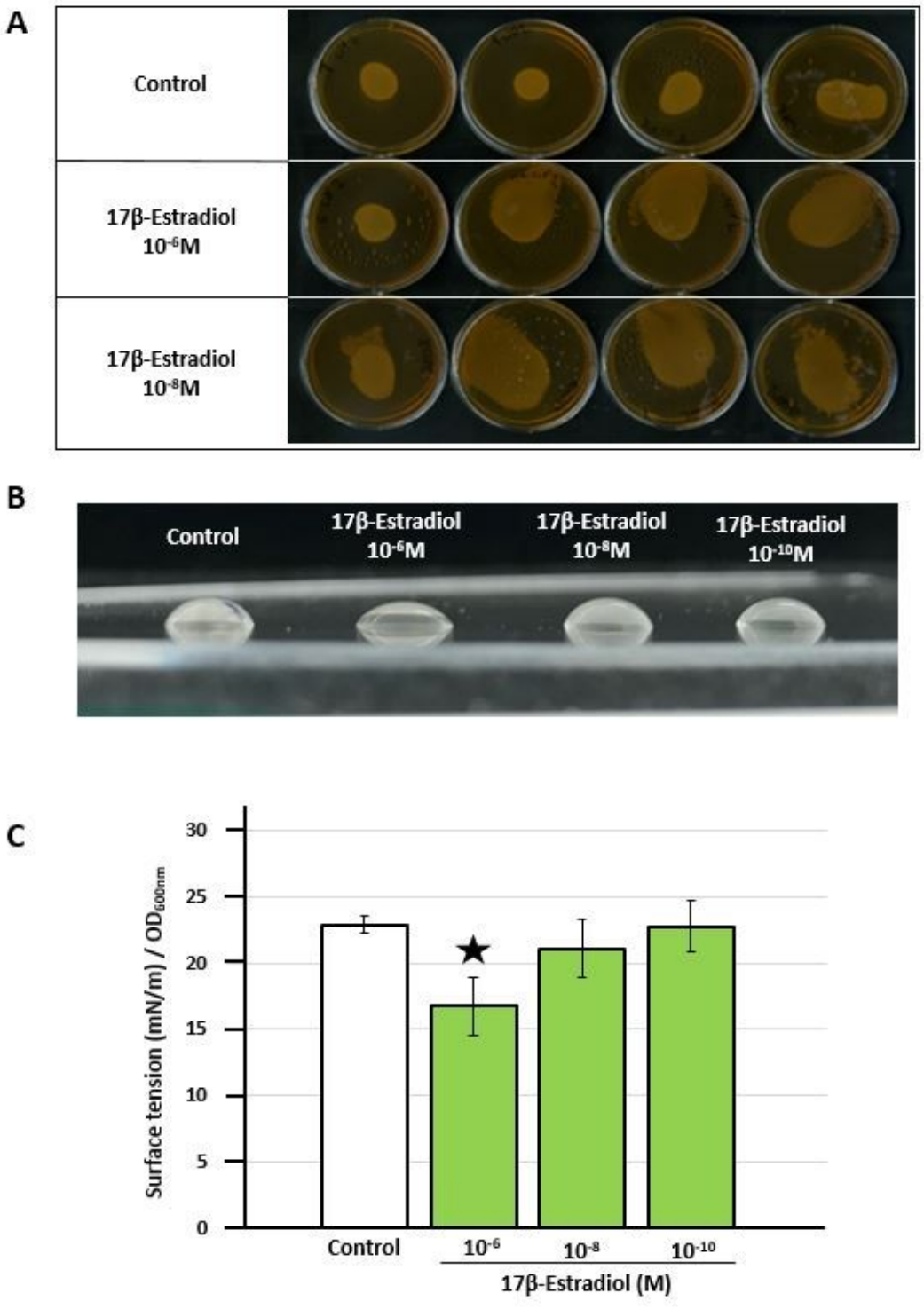

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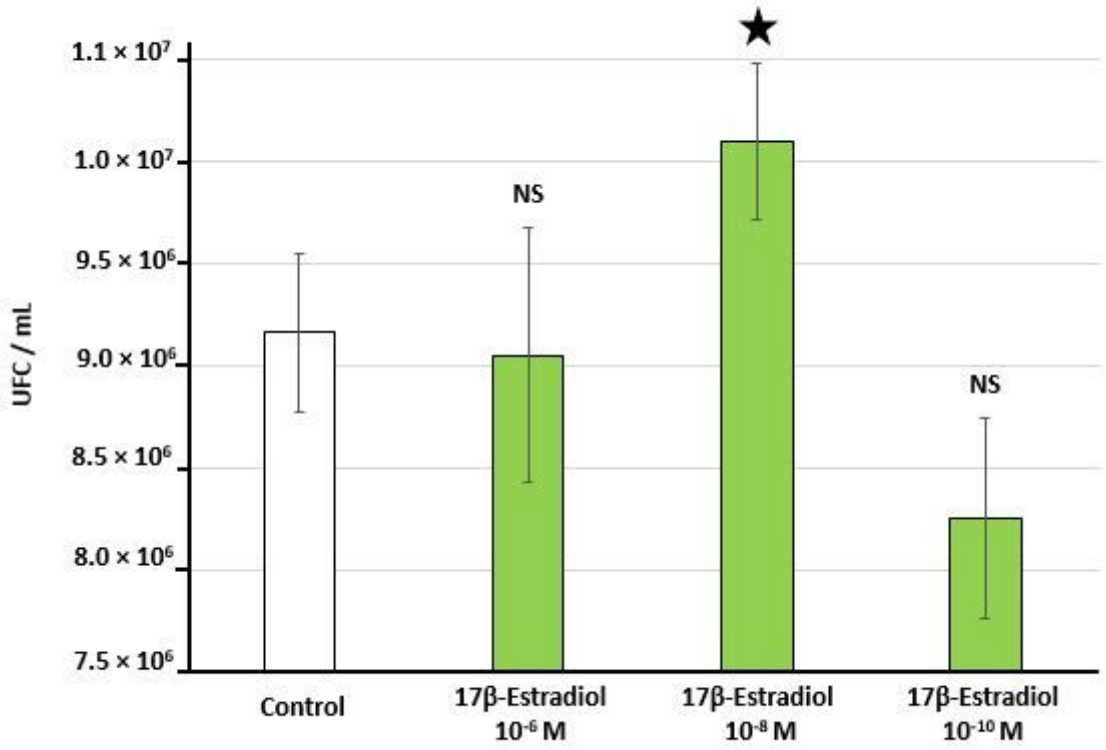

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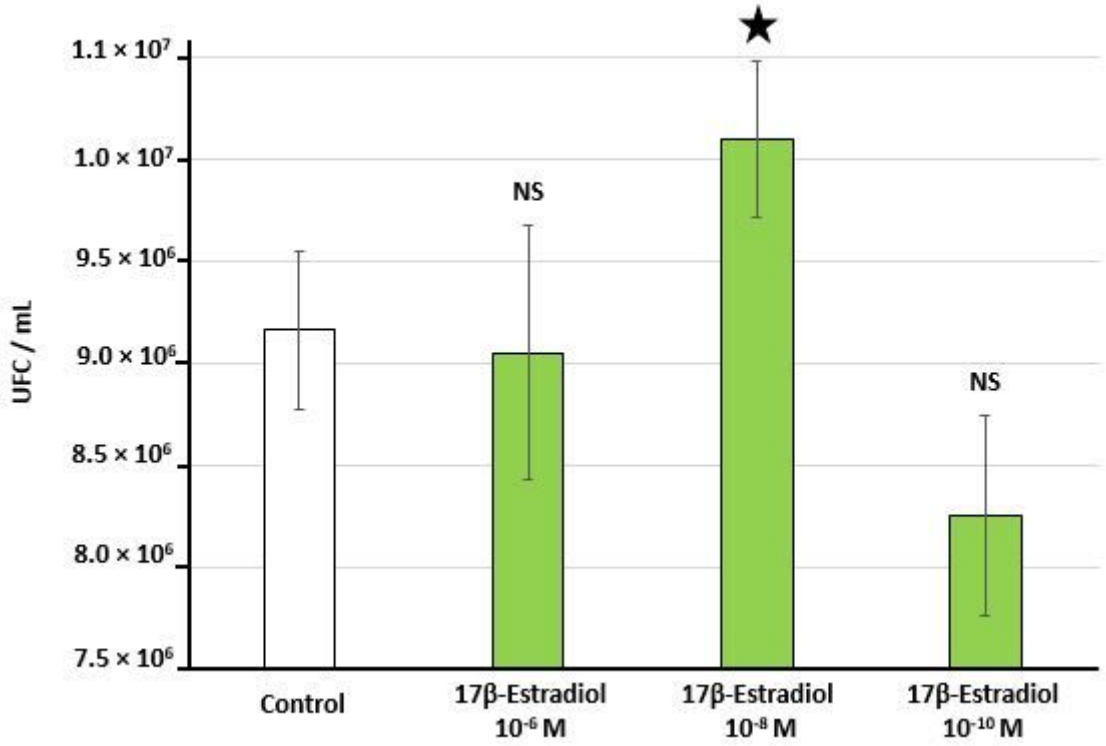
Effect of 17β-estradiol on the adhesion potential to VK2/E6E7 human vaginal cells of Lactobacillus crispatus CIP104459 grown in De Man, Rogosa and Sharpe (MRS) medium. Values are the means ± SEM of three independent studies. (NS: not significant; = $p < 0.05$).



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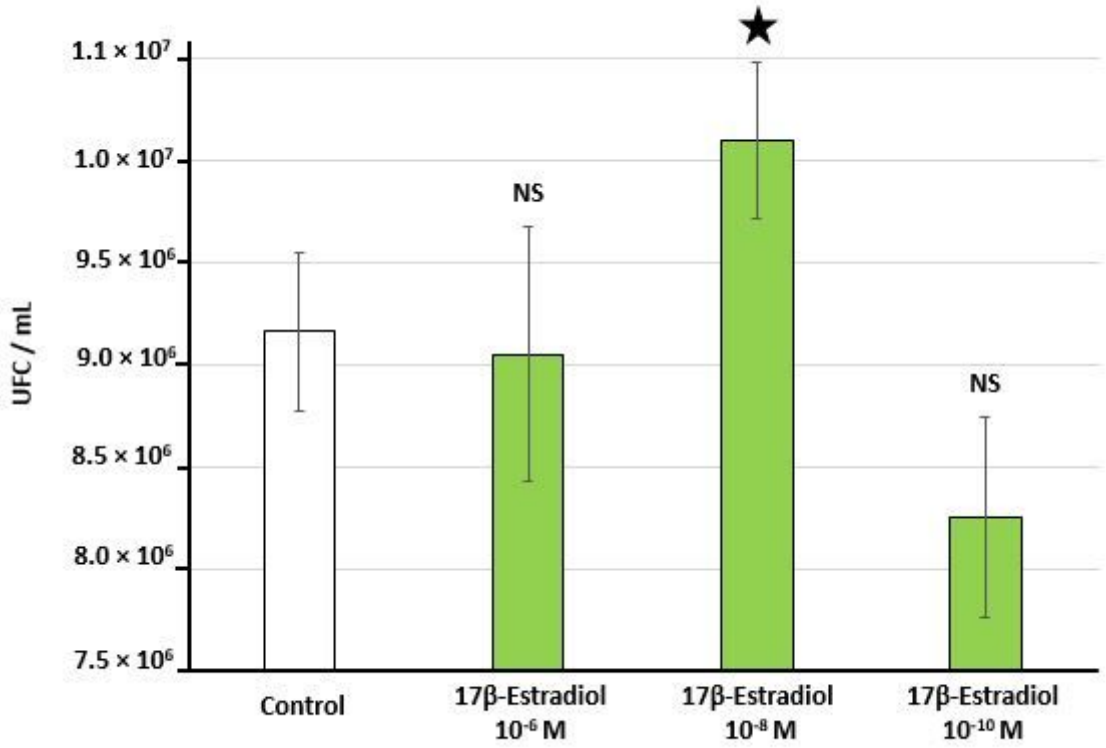

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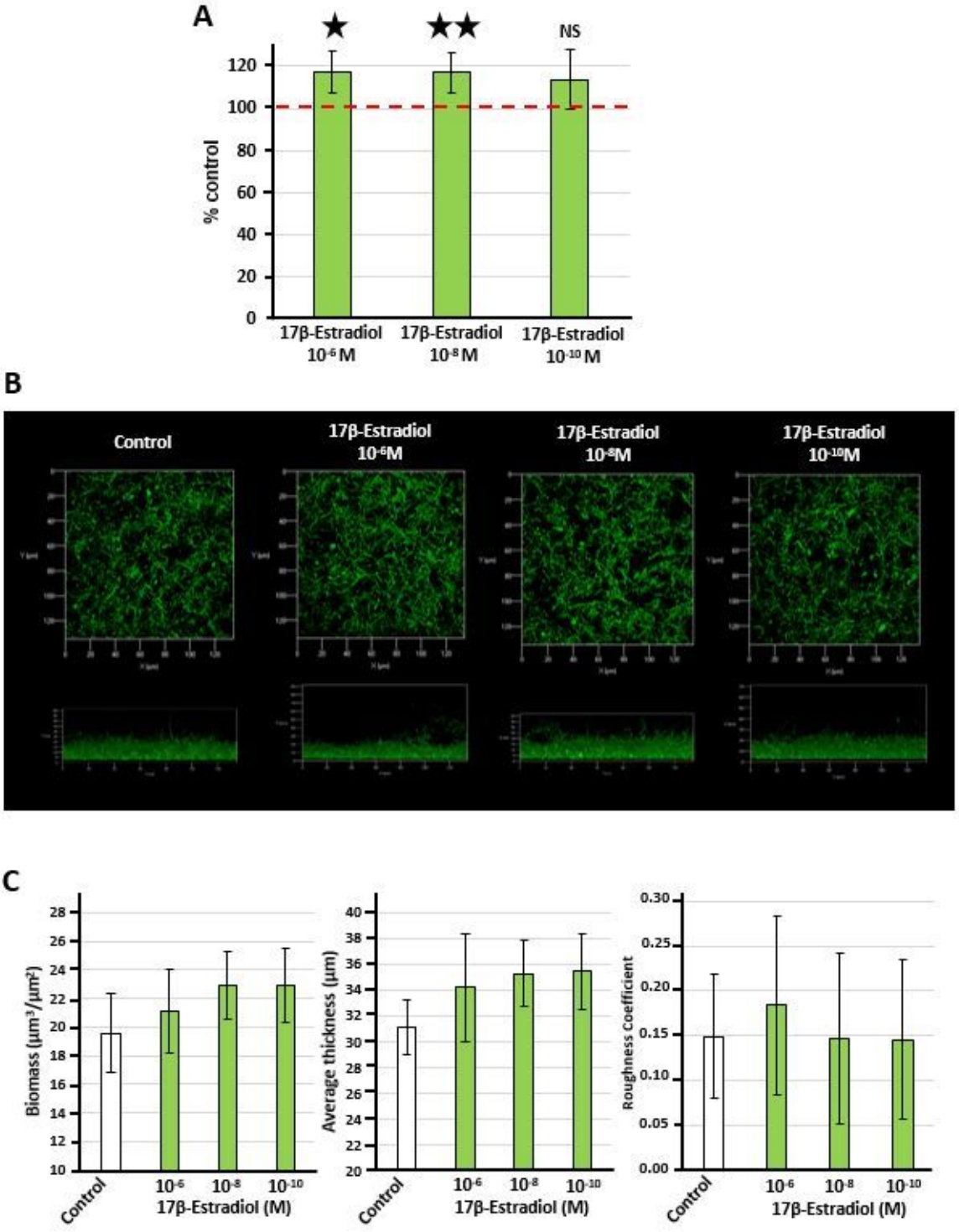

Figure 6

Effect of 17β-estradiol (10-6, 10-8 and 10-10 M) on the biofilm formation activity of Lactobacillus crispatus CIP104459. (A) Biofilm formation of bacteria grown in De Man, Rogosa and Sharpe (MRS) medium in the absence or presence of 17β-estradiol studied by the crystal violet technique. The dotted line indicates the control level (100%). (B) Structure of Lactobacillus crispatus CIP104459 biofilm formed in simulating genital tract secretion (SGTS) medium and visualized by confocal laser scanning

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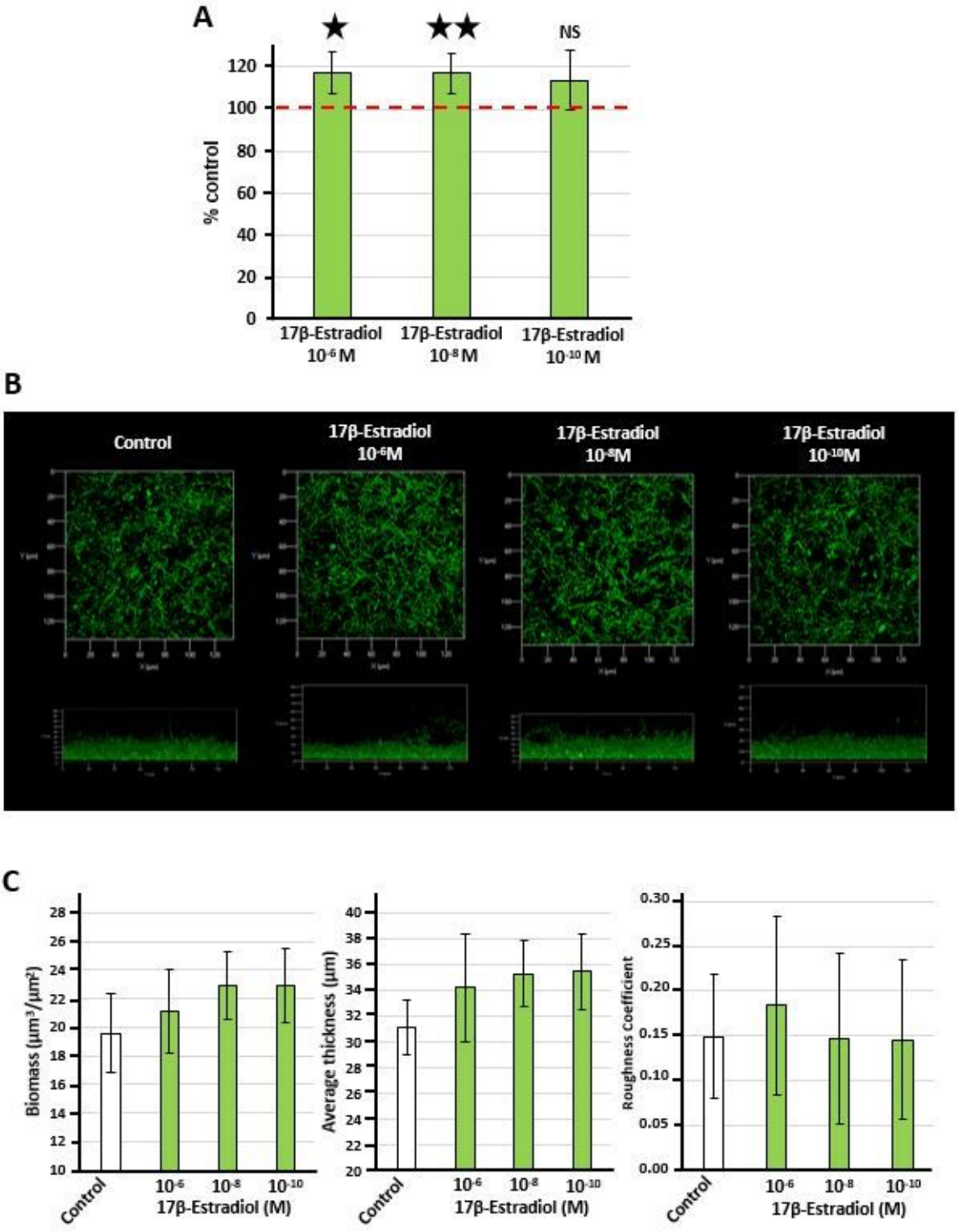

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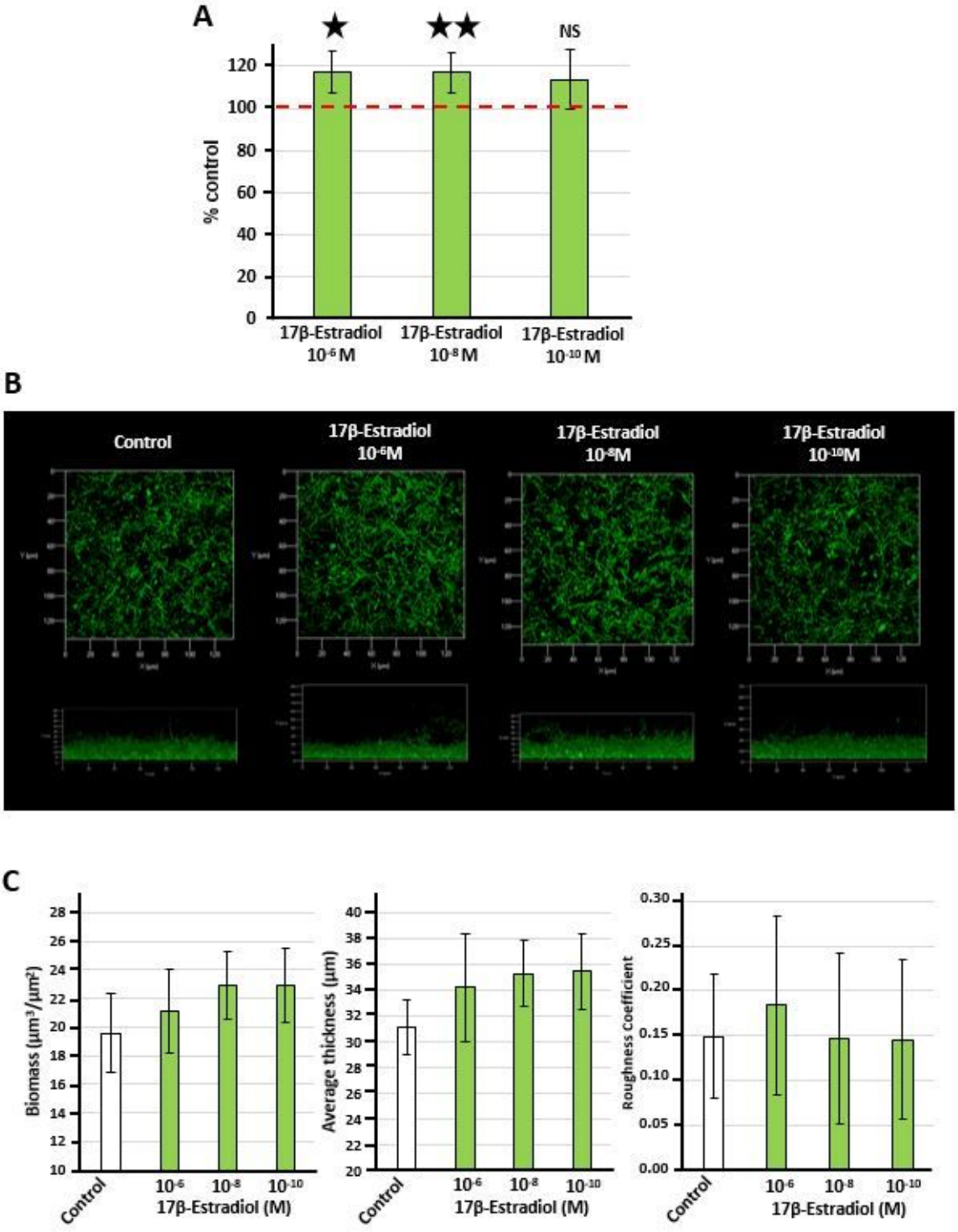

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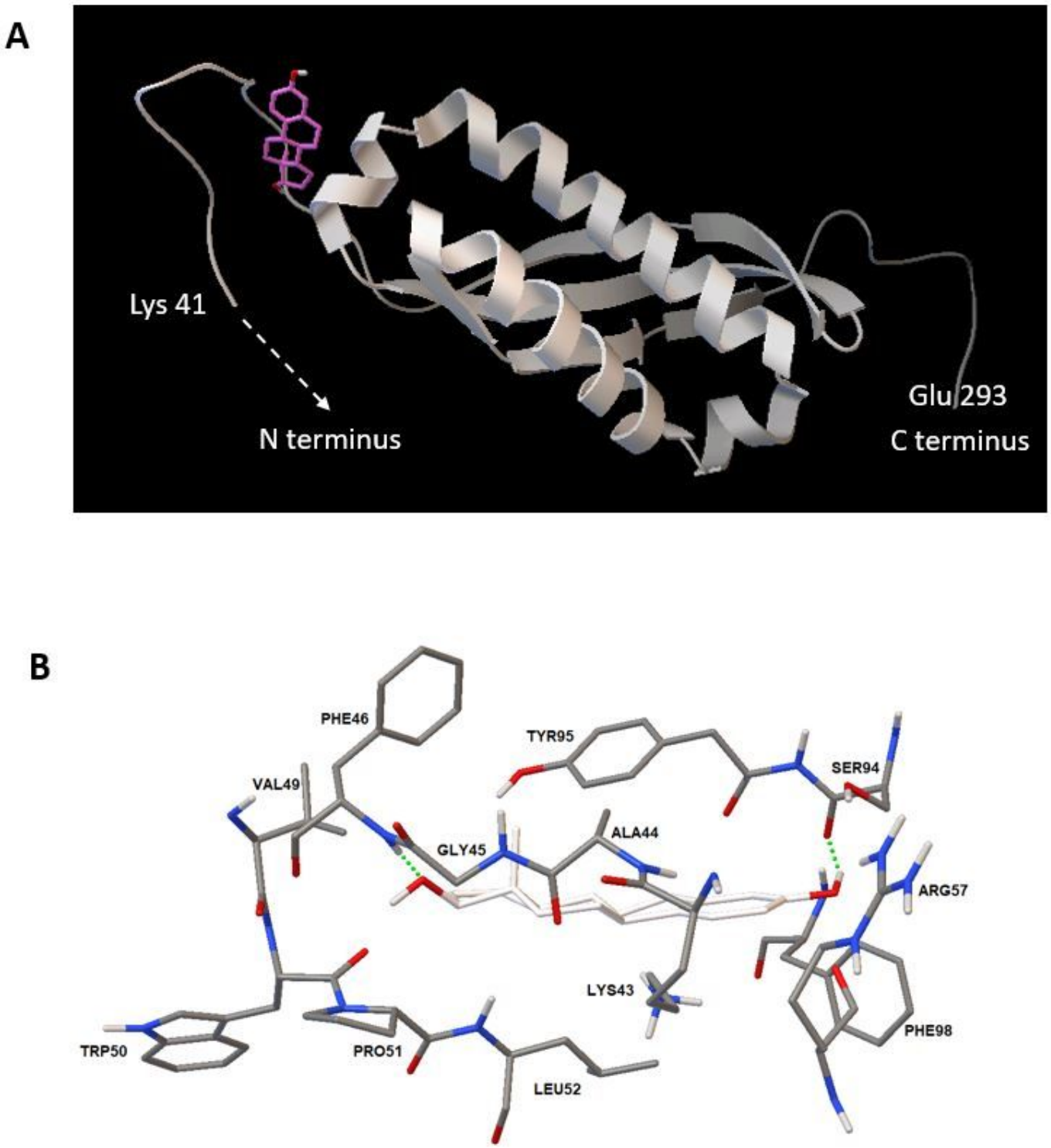
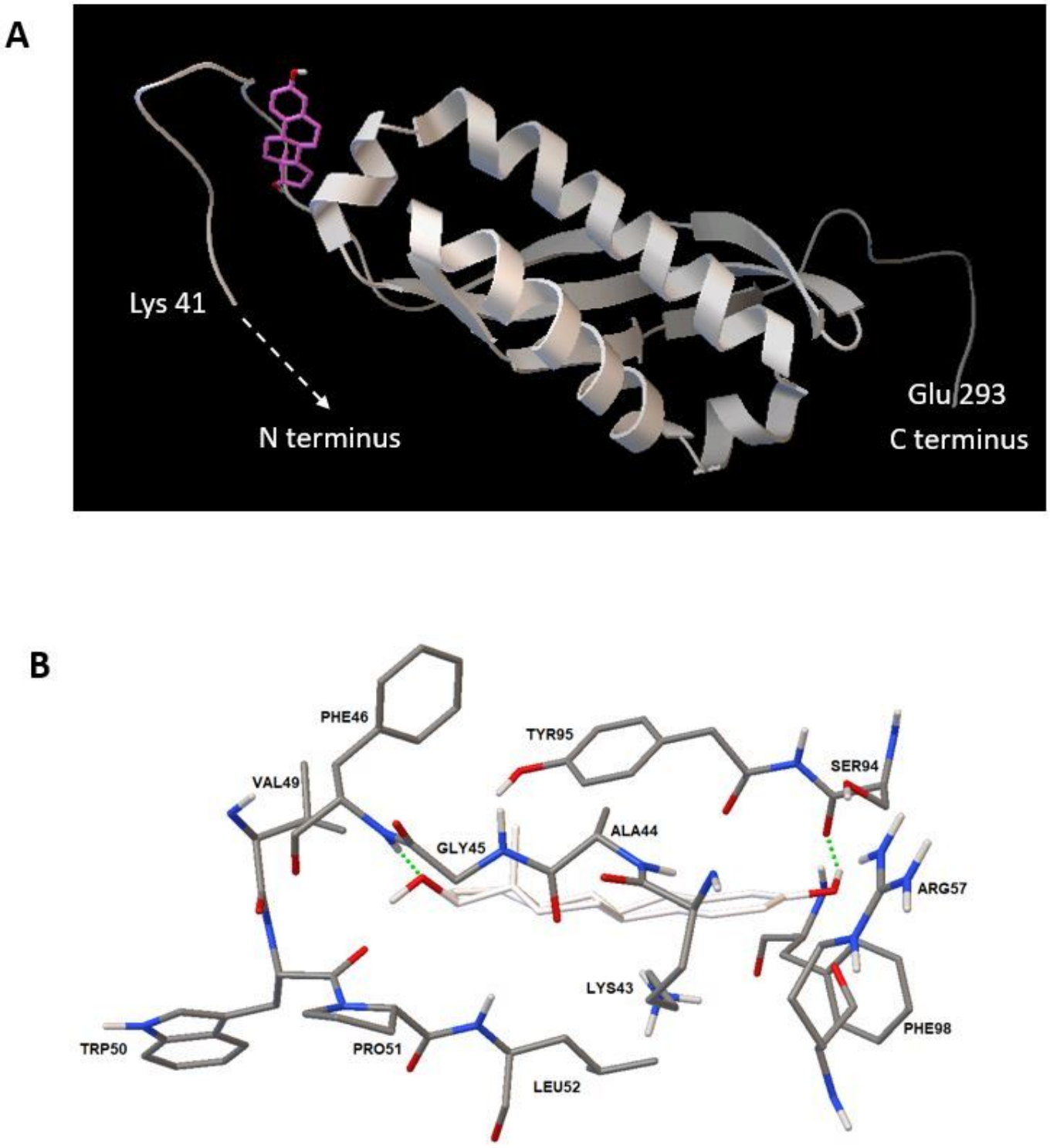


Figure 7

3D modeling of the potential interaction of the Lactobacillus crispatus CIP104459 SPFH domain containing protein (NCBI Reference Sequence: WP_013086692.1) with 17β-estradiol. (A) Illustration of the sub-membrane domain of the L. cristapus SPFH domain containing protein showing its potential interaction with 17β-estradiol (pink). (B) Calculated 17β-estradiol binding site on the L. cristapus SPFH domain containing protein determined under AutoDock 4.2 (17β-estradiol appears in white - hydrogen bonds between 17β-estradiol and amino acids are show as green dotted lines)



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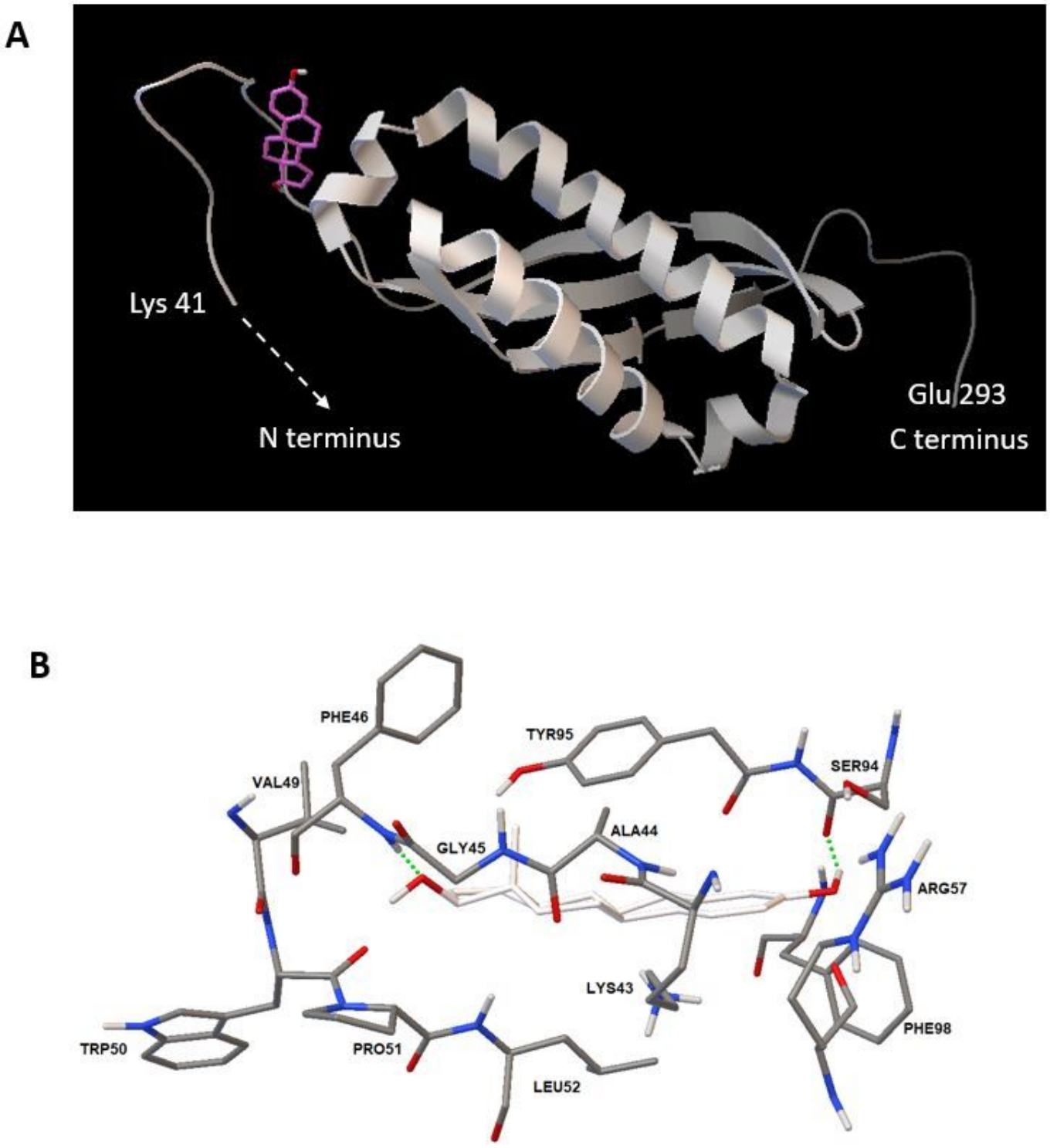

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