LETTER

Swimming bacteria in Poiseuille flow: The quest for active Bretherton-Jeffery trajectories

To cite this article: Gaspard Junot et al 2019 EPL 126 44003

View the article online for updates and enhancements.

Recent citations
- The influence of motility on bacterial accumulation in a microporous channel
  Miru Lee et al
- Upstream swimming and Taylor dispersion of active Brownian particles
  Zhiwei Peng and John F. Brady
- Diffusion properties of self-propelled particles in cellular flows
  Lorenzo Caprini et al
Swimming bacteria in Poiseuille flow: The quest for active Bretherton-Jeffery trajectories

Gaspard Junot\textsuperscript{1}, Nuris Figueroa-Morales\textsuperscript{1(a)}, Thierry Darnige\textsuperscript{1}, Anke Lindner\textsuperscript{1}, Rodrigo Soto\textsuperscript{3}, Harold Auradou\textsuperscript{2} and Eric Clément\textsuperscript{1}

\textsuperscript{1} Laboratoire PMMH-ESPCI Paris, PSL Research University, Sorbonne Université and Denis Diderot 7, quai Saint-Bernard, Paris, France
\textsuperscript{2} Laboratoire FAST, Univ. Paris-Sud, CNRS, Université Paris-Saclay - F-91405, Orsay, France
\textsuperscript{3} Physics Department, FCFM, Universidad de Chile - Av. Blanco Encalada 2008, Santiago, Chile

received 14 March 2019; accepted in final form 27 May 2019
published online 28 June 2019

PACS 47.63.Gd – Swimming microorganisms
PACS 47.63.mf – Low-Reynolds-number motions

Abstract – Using a 3D Lagrangian tracking technique, we determine experimentally the trajectories of non-tumbling \textit{E. coli} mutants swimming in a Poiseuille flow. We identify a typology of trajectories in agreement with a kinematic “active Bretherton-Jeffery” model, featuring an axisymmetric self-propelled ellipsoid. In particular, we recover the “swinging” and “shear tumbling” kinematics predicted theoretically by Zöttl and Stark (\textit{Phys. Rev. Lett.}, 108 (2012) 218104). Moreover using this model, we derive analytically new features such as quasi-planar piecewise trajectories, associated with the high aspect ratio of the bacteria, as well as the existence of a drift angle around which bacteria perform closed cyclic trajectories. However, the agreement between the model predictions and the experimental results remains local in time, due to the presence of Brownian rotational noise.

Understanding the motility and spreading of microorganisms in complex environments, sometimes undergoing significant flow variations, is relevant to many fundamental and technological issues. For instance, this is a crucial question in the context of medicine, as motility can control several physiological functions (\textit{e.g.}, spermatic transport in biological channels \cite{1,2,3}, upstream contamination of urinary tracks \cite{4,5,6} or virulence factors \cite{7}). It is also relevant to technologies of drug delivery \cite{8} and environmental studies aiming to understand the spreading of bio-contaminants in soils \cite{9} or the building of ecological niches \cite{10}.

At the micro-hydrodynamical level, many models aiming to describe the transport processes of micro-swimmers in flow are based on a simple representation initiated by Jeffery \cite{11} and later completed by Bretherton \cite{12}. The Bretherton-Jeffery (B-J) description assesses the changes of orientation of an axisymmetric ellipsoid in a Stokes flow, performing so called Jeffery orbits. The active version of the model (aka “the active B-J model”) is completed with a swimming velocity added to the local flow velocity \cite{13}. In the active B-J model the ellipsoid orientation determines the swimming direction and the orientation dynamics thus directly translate into complex particle trajectories. Passive particles in contrast do not cross streamlines and are merely transported downstream with the flow while tumbling with the velocity gradient. In the presence of Brownian rotational noise, orientation distributions can be determined for passive particles in flows \cite{13-15}. By including noise in the kinematic equations, the active B-J model is the base for many recent statistical-mechanics studies or hydrodynamic dispersion models \cite{13}. This is for example used to describe the emergence of a viscous response for active fluids \cite{13,16} or the mean transport properties of bacteria in microfluidic channels \cite{17-21}. It can also serve to understand the motion of microorganisms in the presence of an external field such as magnetotactic bacteria moving in a flow in the presence of a magnetic field \cite{22,23}, an algae in a light intensity gradient \cite{24} or in a gravity field for bottom-heavy strains \cite{25}.

Interestingly, from an in-depth analysis of these kinematic equations without noise, Zöttl and Stark \cite{26,27} identified mathematical features and associated them with...
the emergence of cycloid trajectories. In some cases the kinematics can be mapped onto a dynamical Hamiltonian problem with conserved constants of motion [26,27]. This model relies on a simplified vision of the bacterial shape and swimming behavior. For instance, the chirality of the bundle responsible of rheotactic effects [28,29] or the flexibility of the flagella [30–32] are neglected.

Despite the importance of this fundamental model, experimental validation and proof of its applicability to bacterial trajectories is still lacking. Tracking experiments of bacteria under flow have already been performed in 3D [33] but no B-J trajectories have been reported. A first experimental observation of these trajectories has been performed by Rusconi et al. [18] but only in the form of a 2D projection. Bacterial trajectories being 3D no full characterization of the dynamics and no direct comparison with the active B-J model could be performed.

In this letter, we investigate experimentally the 3D motion of Escherichia coli (E. coli) bacteria in a plane Poiseuille flow. We use a strain for which the tumbling process is inactivated and reorientation is due to the hydrodynamic shear and Brownian noise. Using a Lagrangian tracking device [34,35], we identify the typology of many experimental 3D trajectories at different flow rates and compare them with the simulated trajectories stemming from the noiseless B-J model. Thanks to the 3D observation of the trajectories, we identify features such as swimming planes and drift angles, providing a better understanding of bacterial transport under flow. We derive several analytic solutions from the B-J model allowing a precise comparison with the experimental observations. Overall, we find good agreement between the experimental tracks and the theoretical predictions of the B-J model. The agreement seems robust despite the possibly more complex shape and swimming properties of real bacteria when compared to the simplified assumptions of an ellipsoid swimmer model. However, this agreement remains local in time due to the cumulative influence of the rotational noise on the trajectory.

**Set-up and protocol.** The measurements take place in a microfluidic channel of rectangular cross-section (height $h = 100 \mu m$, width $W = 600 \mu m$), made in Polydimethylsiloxane (PDMS) using standard soft-lithography techniques. Flow is imposed through the channel via a Nemesys syringe pump (dosing unit Low Pressure Syringe Pump nMESYS 290N and base Module BASE 120N). We set our region of interest in the center of the channel with respect to its width and consider only trajectories which are at least $100 \mu m$ away from the lateral walls.

The channel is visualized using a home-made Lagrangian tracking microscope [34] here used to track fluorescent swimming bacteria as well as passive tracers used for an accurate determination of the fluid velocity profile in the channel. By a visualization based feedback acting on a mechanical (horizontal) and piezoelectric (vertical) stage, the targeted object is kept close to the center of the visualization field and in focus on an inverted microscope (Zeiss-Observer, Z1 with an objectif C-Apochromat 63×/1.2 W). Images of the tracked objects are acquired at 100 Hz with a Hamamatsu Orca-flash 4.0 camera. Simultaneously, the three-dimensional positions of the object are recorded. Figure 1(b) shows velocity profiles $v_x(z)$ obtained by tracking fluorescent beads of diameter 1 $\mu m$. Each dot is the mean velocity of a tracked bead, the dashed lines are parabolic fits used to obtain the maximal velocity at the center of the channel, $V_M$.

The bacteria used here are smooth swimmer mutants of an E. coli (strain CR20, $\Delta$-CheY) that almost never tumble and were transformed with a plasmid coding for a yellow fluorescent protein (YFP). Bacteria are grown overnight at 30°C until the early stationary phase. The growth medium is then removed by centrifuging the culture and removing the supernatant. The bacteria are resuspended in a Motility Buffer (MB) below the very low concentration of $3 \times 10^7$ bacteria per mL, in order to visualize one bacterium at a time and to minimize the interactions between bacteria. The suspension is supplemented with L-serine at 0.08 g/mL and polyvinyl pyrrolidone (PVP) at 0.005%; L-serine maintains good motility for a few hours and PVP is used to prevent bacteria from sticking to surfaces. The solution is also mixed with Percol (1:1) to avoid bacterial sedimentation. The experiments are performed at a temperature of 25°C.

Hundreds of trajectories are recorded at different flow rates $Q$, ranging from 1 to 6 $\mu L/s$, corresponding respectively to $V_M$ between $(28 \pm 1.9) \mu m/s$ and $(168 \pm 5.4) \mu m/s$ and maximal shear rates $\tilde{\gamma}_M = 4V_M/h$ between $(1.12 \pm 0.076) s^{-1}$ and $(6.72 \pm 0.22) s^{-1}$. Here we focus on bulk trajectories that are at least 5 $\mu m$ away from the top and...
Swimming bacteria in flow

Fig. 2: Typology of different 3D bacterial trajectories in Poiseuille flow projected in the shear plane $z$-$x$ for different values of the parameters $A = V_b/4V_M$ and $\beta$. (a) Experimental trajectories: for each type of trajectory (different colors) two experimental trajectories are shown: from left to right: $A = 0.067, 0.062$ (orange), $A = 0.15, 0.057$ (red), $A = 0.072, 0.053$ (light blue) and $A = 0.031, 0.020$ (dark blue). (b) Numerical trajectories: from left to right: $A = 0.068, 0.15, 0.072, 0.031$ and $\beta = 0.95, 0.95, 0.95, 0.80$. 4 types of trajectories are observed: i) beginning and ending at the same wall (red), ii) starting and ending at a different wall (orange), iii) performing cycloid motion in a half-channel (dark blue) and iv) in the whole channel (light blue). For the numerical trajectories the values of $\beta$ have been chosen such as to reproduce qualitatively the experimental trajectories.

The bottom walls. Surface effects are known to modify bacterial trajectories close to channel walls [29,36] but are not the focus of the present paper. They also play a role in setting the initial conditions of bulk trajectories through bacteria take off from the surfaces. The instantaneous swimming velocity is obtained for individual bacteria by removing the local flow velocity from the Lagrangian bacterial velocity. For each track, this instantaneous swimming velocities follow a Gaussian distribution, and its mean gives the swimming velocity $V_b$. The average over all tracks is $(V_b) = (25 \pm 5) \mu m/s$. The bacterium orientation is then obtain by dividing the instantaneous swimming velocity by its magnitude.

Typology of experimental trajectories. – Under flow, a significant number of bacterial tracks were recorded and classified into 4 categories: i) trajectories starting and ending at the same channel wall, ii) trajectories starting from a wall and crossing the mid-plane ($z/h = 1/2$) to reach the opposite wall, iii) trajectories oscillating in a half-channel below or above the mid-plane and iv) trajectories oscillating in the bulk and crossing the mid-plane repeatedly. Figure 2(a) shows examples of such trajectories. The cycloid-like trajectories of type iii) and iv) correspond to the shear tumbling and swinging trajectories predicted theoretically by Zöttl and Stark [27]. Note that trajectories starting and ending at channel walls are frequent in our experiments. As smooth swimmer bacteria spend a significant time at the solid boundaries, many trajectories recorded were indeed initiated at a channel wall.

The active Bretherton-Jeffery model. – Under the B-J assumptions, the body of the microorganism is modeled as an ellipsoid of length $l$ and diameter $e$, swimming at a velocity $V_b\mathbf{p}$. The effective ellipsoid coordinates are its centroid position $\mathbf{r} = (x, y, z)$ with an orientation vector $\mathbf{p} = (\cos \theta, \sin \theta \cos \phi, \sin \theta \sin \phi)$ (see fig. 3). The velocity of the ellipsoid is then the vectorial sum of the swimming velocity and the local flow velocity: $\mathbf{V} = V_b\mathbf{p} + \mathbf{v}$. The Bretherton-Jeffery derivation describes also how an axisymmetric ellipsoid of aspect ratio $r = l/e$ is reoriented in a Stokes flow with a strain-rate tensor $\mathbf{E} = (1/2) [\nabla \mathbf{v} + (\nabla \mathbf{v})^T]$ and a rotation rate tensor $\mathbf{Ω} = (1/2) [\nabla \mathbf{v} - (\nabla \mathbf{v})^T]$. In the absence of rotational noise, the kinetic equation governing $\mathbf{p}$ is: $\dot{\mathbf{p}} = (\mathbf{I} - \mathbf{pp})[(\mathbf{E} + \mathbf{Ω})\mathbf{p}]$, with $\mathbf{I}$ the identity tensor and $\beta = (r^2 - 1)/(r^2 + 1)$ the Bretherton parameter. For a Poiseuille flow $\mathbf{v} = 4V_M(1 - z/h)\mathbf{e}_z$, the trajectories are controlled by the dimensionless parameters $A = V_b/4V_M$ fixing the ratio between the bacterium velocity and the maximal flow velocity and $\beta$. In the following, we rescale all distances by the channel height $h$ and time with $\gamma_M$. The swimmer positions and orientations are then given by five coupled dynamical equations (consistent with the equations presented in [27]) implying three dimensionalized position coordinates $(x(t), y(t), z(t))$:

$$
\begin{align*}
\dot{x} &= A \cos(\theta) + z(1 - z), \\
\dot{y} &= A \sin(\theta) \cos(\phi), \\
\dot{z} &= A \sin(\theta) \sin(\phi),
\end{align*}
$$

and two angular coordinates $\theta$ and $\phi$:

$$
\begin{align*}
\dot{\theta} &= \frac{1}{2} \sin(\phi)[\beta \cos(2\theta) - 1](1 - 2z), \\
\sin(\theta)\dot{\phi} &= \frac{1}{2}(\beta - 1)\cos(\phi) \cos(\theta).
\end{align*}
$$

Fig. 3: Parametrization of an effective ellipsoid swimming in a Poiseuille flow.
Fig. 4: Example of a type iv) swinging trajectory (see fig. 2) computed using $A = 0.04$ and $\beta = 0.95$. In the $y$-$z$ plane (green) the bacterium swims with two nearly constant angles $\phi_c$ or $2\pi - \phi_c$. In the $x$-$y$ plane (blue), one observes the cycloid-like trajectory with a drift angle $\psi$. By rotating the trajectory by the drift angle $\psi$ (computed using the analytical expression given by eq. (3)) around the $z$-axis, the trajectory collapses in the $\lambda$-$z$ plane into a closed orbit (in pink).

Fig. 5: Projection in the $y$-$z$, $x$-$y$ and $z$-$\lambda$ planes of experimental (blue lines) and numerical trajectories (red lines). The numerical trajectories are computed using the parameter $A$ determined experimentally and using as initial conditions the positions and angles of the bacterium in the middle of the track considered. The only fitting parameter is $\beta$. The projection in the $\lambda$-$z$ plane is performed using the value of $\psi$ computed with eq. (3). (a) type ii) trajectory ($\beta = 0.90$ and $A = 0.059$). (b) type iii) swinging trajectory ($\beta = 0.95$ and $A = 0.045$). (c) type iv) shear tumbling trajectory ($\beta = 0.86$ and $A = 0.19$).

Model predictions and comparison to experimental observations. – Using the active B-J model without noise, we first numerically determine typical bacterial trajectories. We then derive closed-form expressions for the phase portraits $z(\theta)$ and $\phi(\theta)$ as well as an analytical expression for the bacterial drift angle $\psi$ with respect to the flow direction. We call $z_0$, $\phi_0$ and $\theta_0$ the set of initial conditions. The model predictions are then compared to experimental observations. Note that surface effects are neglected in our model and quantitative comparisons will only be performed on bacterial trajectories that are at least $5\,\mu$m away from the top and the bottom walls.

Trajectories. As no closed-form solutions are available for the B-J model we solve the model numerically. The numerical trajectories displayed in figs. 2, 4 and 5, are obtained by numerical integration of eqs. (1) and (2) simply using an Euler scheme. Typical experimental observations are reproduced by the numerical trajectories as can be seen from fig. 2 where all trajectory types observed are displayed. The detailed properties of these trajectories are discussed below.

Swimming in planes of nearly constant angles. An important remark can be made from the angle variations $\dot{\phi}$ in eq. (2). For strongly elongated particles, $i.e.$, $\beta \rightarrow 1$, the angle derivative is almost zero unless the angle $\theta$ reaches values of $0$ or $\pi$. This property actually corresponds to a swimmer motion dwelling very close to a plane of constant angle $\phi_c$ until a flip occurs for particle orientations close to...
\( \theta = 0 \) or \( \pi \) in order to set the motion into the mirror plane defined by \( \phi = 2\pi - \phi_0 \). In the \( y-z \) plane perpendicular to the flow direction \( x \), these planes appear as lines of directions \( \phi_0 \) and \( (2\pi - \phi_0) \). The \( \phi_0 \) values are of course fixed by the initial conditions. These fixed planes hosting the bacterial motion, can be seen as a consequence of the absence of shear in the \( y \)-direction. In fig. 4, we display a numerical trajectory of type iv) performing swinging motion, as well as its projection onto various planes. The planes of constant angle \( \phi \) are clearly illustrated by the \( y-z \) projection (in green). Figure 5 shows different experimental and numerical trajectories (type ii), iii) and iv)) projected into the same planes as in fig. 4. From the projections into \( y-z \) (first column), one can clearly observe a tendency for a bacterium to swim in planes of nearly constant angle \( \phi \) and also the presence of subsequent flipping between mirror planes. Hence this good agreement between the experimental observations and the simulations indicates that the smooth E.coli swimmers are well modeled by a \( \beta \) value close to 1, corresponding to very elongated objects.

**Drift angle \( \psi \).** For the numerical trajectories of type iii) and iv), i.e., for bacteria traveling in the bulk without touching the walls, another feature can be noticed. When projected into the \( x-y \) plane (see, for example, fig. 4), these trajectories oscillate around a mean direction different from the flow direction hence defining a “drift angle” \( \psi \). This drift can be seen as the ratio of two displacements, one along or against the flow (the latest resulting from bacteria swimming upstream) and an a transverse displacement solely due to the bacterial activity. The corresponding trajectories are periodic in \( z \) (see fig. 2) and correspond to closed trajectories in the \( (z, \theta, \phi) \) phase-space with a time periodicity \( T \). Due to the bacterium activity and the dependence on \( z \) of the local shear, this period \( T \) will be different from the period of the classical Jeffery orbit. Starting from a point in the \( (z, \theta, \phi) \) phase space, we define the period \( T \) as the time to go back to this point. Since \( \dot{x} \) and \( \dot{y} \) only depend on \( z, \theta \) and \( \phi \) (all periodic functions), one can then define the displacements over one period \( T \), along the flow, \( \Delta x \), and perpendicular to the flow, \( \Delta y \). The expressions of these two displacements are obtained by direct integration of eq. (1) and their ratio yields an expression for the tangent of the drift angle \( \psi \). In the Supplementary Material (SupplementaryMaterial.pdf (SM)), we detail a closed-form derivation for the displacements \( \Delta x \) and \( \Delta y \) and show how an analytical expression for \( \tan \psi \) can be obtained. Then the tangent of the drift angle

\[
\tan \psi = f(A, \beta, z_0, \theta_0, \phi_0),
\tag{3}
\]

is parametrized by the dimensionless numbers of the problem, \( A, \beta \) and the initial trajectory conditions \( z_0, \theta_0, \phi_0 \). From the projection of the numerical trajectory of type iv) into the shear plane \( x-y \) in fig. 4 the drift angle \( \psi \) is clearly identified. We define the \( \mu, \lambda \)-coordinates as shown in fig. 4, respectively along and perpendicular to

the drift direction. We then obtain a remarkable property by projecting the trajectory in the plane \( \lambda-z \) resulting from a rotation around \( z \) by the angle \( \psi \) obtained from the analytical expression (eq. (3)): each 3D B-J trajectory collapses onto a closed orbit in the \( z-\lambda \) plane with a shape depending on the initial conditions of the trajectory and on the parameters \( A \) and \( \beta \). Similar results are observed for the experimental trajectories of type iii) and iv) shown together with corresponding numerical predictions in figs. 5(b) and (c). For all these cases the drift angle \( \psi \) and the closed orbits are clearly visible. Note importantly, that the dependence of the drift angle \( \psi \) on initial conditions \( z_0, \theta_0 \) and \( \phi_0 \) may lead to important consequences for the macroscopic transport properties. For example, our calculations show that the direction of the drift is explicitly dependent on the sign of the angle \( \phi_0 \), which might be selected during the phase of detachment from solid boundaries through non-trivial interaction processes between bacteria and the wall [29,37–39]. Any biased distribution of initial orientations stemming from the boundary conditions will contribute to a net bacterial drift which could add up to the rheotactic contribution due to chirality as proposed by Marcos et al. [28].

**Phase portraits.** The derivation of the phase portraits has previously been performed by Zötll and Stark [27]. Here we chose a different angle parametrization (fig. 3) more suited to highlight the geometrical features we experimentally observe and thus rederive these results. We first calculate the phase portrait \( z(\theta) \). The ratio between \( \dot{z} \) and \( \dot{\theta} \) (eqs. (1) and (2)) yields

\[
\frac{dz}{d\theta} = \frac{2A}{[\beta \cos(2\theta)-1](1-2A)},
\tag{4}
\]

and can be integrated to obtain the relation:

\[
z_\pm = \frac{1 \pm \sqrt{1 + 4B(\cos \theta)}}{2},
\tag{5}
\]

where \( a = \sqrt{\frac{3\beta + 1}{2\beta}} \). The solutions \( z_+ \) and \( z_- \) correspond respectively to sections of trajectories in the upper half \((0.5 < z < 1)\) or in the lower half \((0 < z < 0.5)\) of the channel. We then evaluate the phase portrait \( \phi(\theta) \). By dividing \( \beta \) by \( \theta \) (eqs. (1) and (2)) one obtains \( \sin \theta \frac{d\phi}{d\theta} = \frac{(\beta - 1) \cos(\phi) \cos(\theta)}{\sin(\phi) \beta \cos(2\theta) - 1} \), yielding after integration:

\[
\left[ \frac{\cos \phi}{\cos \phi_0} \right] = \left[ \frac{\tan \theta_0}{\tan \theta} \right] \left[ 1 + r^2 \tan^2 \theta \right] \left[ 1 + r^2 \tan^2 \theta_0 \right],
\tag{6}
\]

Figure 6(a) shows good agreement of an experimental trajectory and the numerical prediction represented in the phase portraits. In addition, from the phase portraits displayed in figs. 6(a)–(c), we can rationalize the prominence of trajectories staying close to a plane \( \phi = \phi_0 \) as long as the angle \( \theta \) is not close to 0 or \( \pi \) (i.e., \( \sin \theta \) going to zero). Then, in this last case, the model predicts a shift
Fig. 6: Phase portraits. The phase lines in green are obtained using eqs. (4) and (6), blue line: experimental data, red and black lines are, respectively, numerical simulations without and with rotational noise projected into the phase space. (a) Phase portraits \((A = 0.059 \text{ and } \beta = 0.90)\). (b) and (c): phase portraits \((A = 0.0625 \text{ and } \beta = 0.97)\). The trajectories in black show two different realizations of a numerical trajectory with noise \((D_r = 1/47 \text{s}^{-1})\) simulated using the same initial conditions and parameters as for the red trajectory without noise. In panel (b) simulations with and without noise remain very close, whereas in panel (c) an important difference is observed.

from the initial plane to the mirror plane (see fig. 3). As already noticed this is indeed a robust feature observed experimentally for many trajectories.

Influence of orientational noise. – For all trajectories observed experimentally, the features revealed by the active 3D B-J model have been recovered semi-quantitatively. However, even for the smooth swimmer strain used here, a quantitative agreement between simulation and experiments is only local in time. Indeed, after a relatively short observation period experimental trajectories deviate systematically from the numerical predictions, even for bacterial trajectories that remain far from the channel walls. We attribute this deviation to the presence of orientational noise in the experiment. Such erratic behavior will be crucial in the dispersion mechanism along the direction perpendicular to the flow. The drift angle strongly depends on the initial conditions and a bias in the initial orientation as for example induced by interactions with surfaces might lead to bacteria drift into specific directions.

Crucial questions remain concerning the reorientation of the swimming angles due to rotational noise, which contributes to the hydrodynamic dispersion process (in the real \(x-y-z\) space). Here we have shown that the randomization process observed in the phase space is consistent, at least in magnitude, with a rotational Brownian motion, for an effective ellipsoid. It is, however, possible that other sources of randomization come into play such as the bundle flexibility [41] partial unbundling due to shear, wiggling effects [42] or reorientation due to the chirality of the bacteria flagella [28].

The authors thank Dr Reinaldo García García for useful discussions. This work was supported by the ANR grant “BacFlow” ANR-15-CE30-0013 and the Franco-Chilean EcosSud Collaborative Program C16E03. NF-M thanks the Pierre-Gilles de Gennes Foundation for financial support. AL and NF-M acknowledge support from the
REFERENCES